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PROLYL OLIGOPEPTIDASE AND ALPHA-SYNUCLEIN IN THE REGULATION OF NIGROSTRIATAL DOPAMINERGIC NEUROTRANSMISSION

Ulrika Julku

DOCTORAL DISSERTATION

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ABSTRACT

Dopamine is one of the main neurotransmitters in the brain. Dopaminergic signalling regulates reward, memory, attention and motor functions. In the synapses of dopaminergic neurons, dopamine transporter (DAT) re-uptakes dopamine into the presynaptic nerve terminals after dopamine release terminating the dopaminergic signal and acting as one of the main regulators for kinetics of dopaminergic neurotransmission. Loss of dopaminergic neurons in the nigrostriatal pathway and protein aggregates called Lewy bodies are the main pathological findings in Parkinson´s disease. Lewy bodies are mainly composed of a protein called α-synuclein. The physiological role of α-synuclein has remained unclear but it has been suggested that the main function is regulation of dopaminergic neurotransmission since α-synuclein has been shown to participate in the regulation of dopamine synthesis, storage, release, and metabolism. α-synuclein-regulated functions in dopaminergic signaling are described in the literature review of this thesis.

Prolyl oligopeptidase (PREP) is a serine protease that binds to α-synuclein and induces its aggregation. PREP inhibitors have beneficial effects in cellular and *in vivo* models of Parkinson´s disease by reducing α-synuclein aggregates and oligomers, and improving motor functions. Additionally, PREP inhibitors alter striatal dopamine level in mice and rats, and decrease immunoreactive DAT in the mouse striatum suggesting that PREP could have an effect on dopaminergic function.

The aim of this study was to characterize the role of PREP in dopaminergic signaling and the effect of α-synuclein in PREP-mediated changes of the dopaminergic system. In the first study, the effect of PREP and α-synuclein on DAT phosphorylation and function was studied in DAT transfected HEK-293 cells. PREP altered DAT function and dopamine uptake, but the changes were not dependent on ERK phosphorylation or PKC activity. α-synclein had an effect on DAT phosphorylation in the absence of PREP but this was also independent of phosphorylation of ERK indicating that both α-synuclein and PREP are able to modulate DAT function via an ERK-independent mechanism.

In the second study, the role of PREP in dopaminergic signaling was characterized in the nigrostriatal pathway of mouse. The influence of PREP was investigated by comparing the dopaminergic function of PREP knock-out mice and wild-type littermates. Lack of PREP elevated extracellular dopamine concentration, delayed re-uptake of dopamine, and increased phosphorylation of DAT in the mouse striatum indicating that PREP is able to regulate DAT function by modulating phosphorylation and localization of DAT.

The effect of PREP inhibition on dopaminergic function, behavior, and α-synuclein in a Parkinson´s disease mouse model was investigated in the third study. Overexpression of α-synuclein was induced by supranigral

microinjection of AAV-α-synuclein and mice were treated with the PREP inhibitor KYP-2047 after the onset of the behavioral symptoms. KYP-2047 treatment did not restore α-synuclein-induced reduction in striatal dopamine but behavioral improvement and reduction in α-synuclein oligomers indicated restoration of dopamine release and recycling.

The aim of the fourth study was to investigate if α-synuclein-induced toxicity in the nigrostriatal pathway is dependent on PREP expression. The main finding was that α-synuclein toxicity was reduced in the absence of PREP and restoration of PREP expression increased toxicity in the behavioral tests. However, nigrostriatal dopamine level was not affected suggesting that lack of PREP protects dopamine release and recycling from α-synuclein-induced toxicity.

In conclusion, PREP regulates DAT function in cells and in the mouse nigrostriatal pathway, but the mechanism is not dependent on ERK and PKC activation. Deletion of PREP or PREP inhibition do not have effects on α-synuclein-induced dopaminergic cell loss, but they are able to restore behavior and dopaminergic function in the mouse brain suggesting that PREP inhibitors could provide a novel treatment for Parkinson´s disease.

TIIVISTELMÄ

Dopamiini kuuluu aivojen tärkeimpiin hermovälittäjäaineisiin, ja se säätelee mm. tahdonalaisia liikkeitä ja mielihyvää. Parkinsonin taudissa dopamiinihermosolut tuhoutuvat nigrostriataaliradaksi kutsutulla aivoalueella, mikä aiheuttaa vapinaa ja liikehäiriöitä. Nykyisillä hoitomenetelmillä, jotka perustuvat aivojen dopamiinin korvaamiseen, voidaan lievittää taudin oireita, mutta ei hidastaa tai pysäyttää sairauden etenemistä. Terveissä aivoissa α-synukleiiniksi kutsuttu proteiini osallistuu dopamiinin muodostumisen, varastoitumisen, vapautumisen ja hajoamisen säätelyyn, mutta Parkinsonin tautia sairastavien aivoissa α-synukleiinista muodostuu proteiinikertymiä, jotka ovat haitallisia solujen normaalille toiminnalle. Prolyylioligopeptidaasi (PREP) on entsyymi, joka lisää α-synukleiinikertymien muodostumista, ja aiemmissa tutkimuksissa on selvinnyt, että PREP-entsyymin toimintaa estämällä voidaan vähentää α-synukleiinikertymiä Parkinsonin taudin solu- ja eläinmalleissa. PREP-estäjien on myös havaittu aiheuttavan muutoksia dopamiinijärjestelmän toimintaan aivojen nigrostriataaliradassa hiirillä ja rotilla.

Tämän väitöskirjatyön tarkoituksena oli tutkia tarkemmin PREP:n roolia aivojen nigrostriataaliradan dopamiinijärjestelmässä. Lisäksi tutkittiin, että voidaanko PREP-entsyymin toimintaa estämällä korjata α-synukleiinin aiheuttamia hermosoluvaurioita Parkinsonin taudin hiirimallissa ja suojaako PREP:n poistaminen hiiriä α-synukleiinin vahingollisilta vaikutuksilta.

Tärkeimpänä löydöksenä oli PREP:n osallistuminen dopamiininkuljettajaproteiinin toiminnan säätelyyn sekä soluissa että hiiren aivoissa. Solukokeet kuitenkin osoittivat, että PREP ei säätele dopamiininkuljettajaproteiinin toimintaa sen keskeisimpänä pidettyjen säätelijöiden kautta. Parkinsonin taudin hiirimallissa PREP-estäjällä saatiin korjattua käytösmuutoksia ja dopamiinijärjestelmän toimintaa, vaikka hoidolla ei ollutkaan vaikutusta aivojen dopamiinisolutuhoon. Lisäksi havaittiin, että PREP:n puuttuminen vähensi α-synukleiinin haitallisia vaikutuksia hiirillä. Yhteenvetona näiden löydösten perusteella voidaan todeta, että PREP osallistuu aivojen dopamiinijärjestelmän säätelyyn, ja että PREP-estäjät ovat lupaava uusi keino Parkinsonin taudin hoitoon.

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Helsinki, April 2019

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Julku, U.H.**, Jäntti, M., Svarcbahs, R., and Myöhänen, T. T. (2019) Prolyl oligopeptidase regulates dopamine transporter phosphorylation in a PKC and ERK independent manner. Manuscript submitted.

II **Julku, U. H.**, Panhelainen, A. E., Tiilikainen, S. E., Svarcbahs, R., Tammimäki, A. E., Piepponen, T. P., Savolainen, M. H. and Myöhänen, T. T. (2018) Prolyl oligopeptidase regulates dopamine transporter phosphorylation in the nigrostriatal pathway of mouse. Molecular neurobiology, 55, 470-482.

III Svarcbahs, R.*, **Julku, U. H.*** and Myöhänen, T. T. (2016) Inhibition of Prolyl Oligopeptidase Restores Spontaneous Motor Behavior in the α-Synuclein Virus Vector–Based Parkinson's Disease Mouse Model by Decreasing α-Synuclein Oligomeric Species in Mouse Brain. Journal of Neuroscience, 36, 12485-12497. *equal contribution

IV Svarcbahs, R., **Julku, U. H.**, Norrbacka, S. and Myöhänen, T. T. (2018) Removal of prolyl oligopeptidase reduces alpha-synuclein toxicity in cells and *in vivo*. Scientific Reports, 8, 1552.

The publications are referred in the text by their roman numerals. Supplementary results are also presented for the studies I, II and IV. Reprints were made with the permission of copyright holders.

The division of labour in the publications.

Study I	
Study design	UHJ, TTM, MJ
Laboratory work and data analysis:	
Creation of PREPko cell line	RS
Western blotting, DA uptake	UHJ
PKC activity assay	MJ
Immunofluorescence staining	TTM
Manuscript writing:	
Original draft	UHJ
Review and editing	TTM, MJ, RS
Study II	
Study design	UHJ, AET, AEP, TPP, TTM
Laboratory work and data analysis:	
Creation of viral vectors	MHS
Surgeries	UHJ, SET, RS
Microdialysis, tissue HPLC, behavioral tests	UHJ, SET
Fast-scan cyclic voltammetry	AEP
Western blotting	SET, TTM
Publication writing:	
Original draft	UHJ
Review and editing	AET, AEP, TPP, RS, MHS, TTM
Study III	
Study design	RS, UHJ, TTM
Laboratory work and data analysis:	
Surgeries and cylinder test	UHJ, RS
Microdialysis and tissue HPLC	UHJ
Immunohistochemistry	RS
Publication writing:	
Original draft	RS
Review and editing	TTM, UHJ
Study IV	
Study design	RS, UHJ, TTM
Laboratory work and data analysis:	
Creation of PREPko cell line	RS
Surgeries	UHJ, RS
Behavioral tests	RS
Microdialysis and tissue HPLC	UHJ
Cell work, except Western blotting	RS ,SMN
Western blotting	UHJ, RS
Publication writing:	
Original draft	RS
Review and editing	UHJ, TTM

ABBREVIATIONS

5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine
AADC	Aromatic amino acid decarboxylase
AAV	Adeno-associated virus
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
aSyn	α-Synuclein
BCA	Bicinchoninic acid
CaMKII	Calcium-calmodulin-dependent protein kinase II
COMT	Cathecol-O-methyl transferase
DA	Dopamine
DAergic	Dopaminergic
DAT	Dopamine transporter
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco´s modified Eagle´s medium
DOPAC	3,4-dihydroxyphenyl acetic acid
DOPAL	3,4-dihydroxyphenyl acetaldehyde
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric acid
GBA	Glucocerebrosidase
GFP	Green fluorescence protein
HEK	Human embryonic kidney
HPLC	High-performance liquid chromatography
HVA	Homovanillic acid
LRRK2	Leucine-rich repeat kinase 2
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MPP^+	1-Methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson´s disease
pDAT	Phosphorylated DAT
pERK	Phosphorylated ERK
PINK1	PTEN-induced putative kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PLD2	Phospholipase D2
PMA	Phorbol 12-myristate 13-acetate
PP2A	Protein phosphatase 2A

PREP	Prolyl oligopeptidase
PREPko	PREP knock-out
ROS	Reactive oxygen species
SEM	Standard error of mean
SN	Substantia nigra
SNAP-25	Synaptosomal nerve-associated protein 25 kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor-attached receptor
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STR	Striatum
TH	Tyrosine hydroxylase
VAMP2	Vesicle-associated membrane protein 2
VMAT	Vesicular monoamine transporter
VEH	Vehicle
Wt	Wild-type

1 INTRODUCTION

Dopamine (DA) is one of the main neurotransmitters in the brain. It is a chemical messenger involved in reward (Schultz 1998), motivation (Salamone & Correa 2012), memory (Luciana *et al.* 1998), attention (Nieoullon 2002), and motor functions (Matsumoto *et al.* 1999, Hikosaka *et al.* 2002). There are several DAergic pathways in the brain, but this thesis focuses on the nigrostriatal pathway which has a central role in Parkinson´s disease (PD) pathology (Ehringer & Hornykiewicz 1960). PD is a neurodegenerative disease that has resting tremor, slowness of movements, and rigidity as typical motor symptoms (Kalia & Lang 2015). The main pathological findings in PD are loss of DAergic neurons in the nigrostriatal tract and protein aggregates called Lewy bodies. Alpha-synuclein (aSyn) is the main component in Lewy bodies (Spillantini *et al.* 1997). The physiological role of aSyn has remained unclear, but modulation of DAergic neurotransmission has been suggested as the main role for aSyn since aSyn has been shown to participate in regulation of DAergic function by modulating synthesis, storage, release and re-uptake of DA (Butler *et al.* 2017). Dysfunction of these mechanisms could possibly mediate DAergic cell loss in PD. The literature review in this thesis will focus on the interplay between aSyn and DAergic neurotransmission.

aSyn has a monomeric or tetrameric form in native conditions (Eliezer *et al.* 2001), but as an intrinsically disordered protein aSyn is prone to misfolding and aggregation, and there are numerous studies showing that aSyn oligomers or fibrils are toxic and they are able to induce DAergic cell loss in PD (Pieri *et al.* 2016, Conway *et al.* 1998, Tsigelny *et al.* 2012). PD is idiopathic in most of the cases but there are several PD-linked gene mutations in aSyn coding SNCA gene, such as duplication or triplications (Polymeropoulos *et al.* 1997, Singleton *et al.* 2003, Chartier-Harlin *et al.* 2004) and several known point mutations that predispose people to PD. These gene mutations induce formation of toxic forms of aSyn and they have also been used in preclinical PD models. There are also other PD linked gene mutations in several different genes such as leucine-rich repeat serine/ threonine- protein kinase 2 (LRRK2), glucocerebrosidase (GBA), vacuolar protein sorting-associated protein 35 (VPS35), PTEN-induced putative kinase 1 (PINK1), Parkin, and DJ-1 amongst many others (Kalia & Lang 2015).

Prolyl oligopeptidase (PREP) is a highly conserved serine protease (Venäläinen et al. 2004, Kaushik & Sowdhamini 2014) that accelerates the aggregation of aSyn (Brandt *et al.* 2008). PREP inhibitors have shown beneficial effects by reducing aSyn aggregates and toxic oligomers, and enhancing motor functions in Parkinson´s disease models (Savolainen *et al.* 2014, Myöhänen *et al.* 2012, Dokleja *et al.* 2014). PREP is also involved in regulation of the nigrostriatal DAergic neurotransmission (Jalkanen *et al.* 2012, Savolainen et al. 2014), and it has been shown that PREP inhibitors are

able to alter nigrostriatal DA level and DAT immunoreactivity in mice and rats (Jalkanen et al. 2012, Savolainen et al. 2014). The role of PREP in the regulation of the nigrostriatal DAergic system and DAT function was studied further in the results part of this thesis. The interplay between PREP, aSyn, and DAT was also studied to investigate if aSyn-induced changes in DAT function can be modulated by PREP.

2 REVIEW OF THE LITERATURE

2.1 OVERVIEW OF BRAIN DOPAMINERGIC SYSTEM

DA was first synthesized in 1910 (Barger & Dale 1910) and its neurotransmitter properties and localization in the brain were found in 1957 (Carlsson *et al.* 1957, Montagu 1957, Carlsson *et al.* 1958). Subsequent studies have shown that DA is one of the main neurotransmitters. The main pathways are the nigrostriatal pathway, mesocortical pathway, mesolimbic pathway, and tuberoinfundibular pathway (Figure 1). This thesis review mainly focuses on the nigrostriatal pathway which controls movements and motor skill learning. In this pathway, the DAergic cell bodies are located in the substantia nigra (SN) and projecting to the dorsal striatum (STR). Loss of nigrostriatal DAergic neurons is one of the main pathological finding in Parkinson´s disease (PD), and the main reason for the motor symptoms of the disease. Impaired DAergic signaling is also involved in several other neurological diseases such as Huntington´s disease, schizophrenia, attention deficit/hyperactivity disorder (ADHD) and psychostimulant drug addiction.

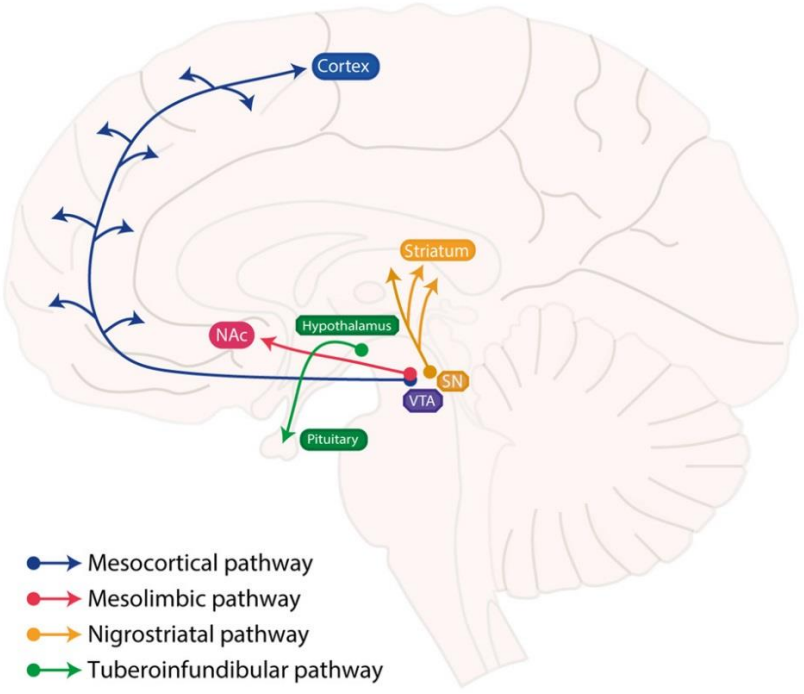

Figure 1 DAergic pathways in the brain. Adapted by permission from Springer Nature: Cellular and Molecular Neurobiology, Dopamine: Functions, Signaling, and Association with Neurological Diseases, Klein M. O., Battagello D. S., Cardoso A. R. et al, Vol 39, pages 31-59, copyright (2018).

2.1.1 DOPAMINE SYNTHESIS AND METABOLISM

The precursor for DA, tyrosine, is coming from ingested protein (Fernstrom & Fernstrom 2007). Substantial biosynthesis of DA also happens outside the brain by mesenteric organs (Eisenhofer *et al.* 1997), but this review focuses on DA synthesis and metabolism in the central nervous system. The classical pathway for DA biosynthesis was found in 1939 (Blaschko 1939). DA is synthesized in the cytosol of catecholaminergic cells in the brain where tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis, converts tyrosine to levodopa using tetrahydrobiopterin, oxygen (O_2), and iron (Fe^{2+}) as cofactors (Nagatsu *et al.* 1964). TH is widely used as a marker for DAergic cells in brain research since it is mainly localized in the catecholaminergic cells (Weihe *et al.* 2006). Aromatic amino acid decarboxylase (AADC) converts levodopa to DA having pyridoxal phosphate as a cofactor (Christenson *et al.* 1970). In addition to the main synthesis pathway, DA can be synthesized by a cytochrome P450-mediated pathway, but the contribution to the total DA synthesis is low in normal conditions (Hiroi *et al.* 1998, Bromek *et al.* 2011, Bromek *et al.* 2010).

After synthesis, DA is stored in presynaptic vesicles by vesicular monoamine transporter 2 (VMAT2) in the DAergic cells or it is further converted to adrenaline and noradrenaline in adrenergic and noradrenergic cells (Eiden & Weihe 2011, Udenfriend & Wyngaarden 1956, Weinshilboum *et al.* 1971). DA is sensitive to oxidation in a non-acidic environment, but VMAT2 protects neurons from oxidative stress by storing DA in the acidic vesicles (Guillot & Miller 2009). Excitation of DAergic neurons leads to release of DA into the synaptic cleft from the presynaptic vesicles by exocytosis. Thereafter, DA can interact with postsynaptic DA receptors or regulatory presynaptic DA autoreceptors. DAergic signaling ends when extracellular DA is removed from the synaptic cleft by reuptake of DA to the presynaptic nerve terminals or by degradation in the glial cells (Meiser *et al.* 2013). VMAT2 recycles DA to vesicles but excess DA, accumulating to the cytosol, is degraded by monoamine oxidase (MAO) to hydrogen peroxide and to the reactive 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is preferentially converted to 3, 4-dihydroxyphenylacetic acid (DOPAC) by the enzymes aldehyde dehydrogenase (ALDH) or alcohol dehydrogenase (ADH). DA is also taken into the glial cells from the synaptic cleft and degraded by MAO or by catechol-O-methyl transferase (COMT). COMT transfers DA into 3-methoxytyramine and it is also able to degrade DOPAC further into homovanillic acid (HVA). DA and its metabolites are then conjugated with sulphates and glucuronides before they are excreted to urine (Buu *et al.* 1981, Uutela *et al.* 2008).

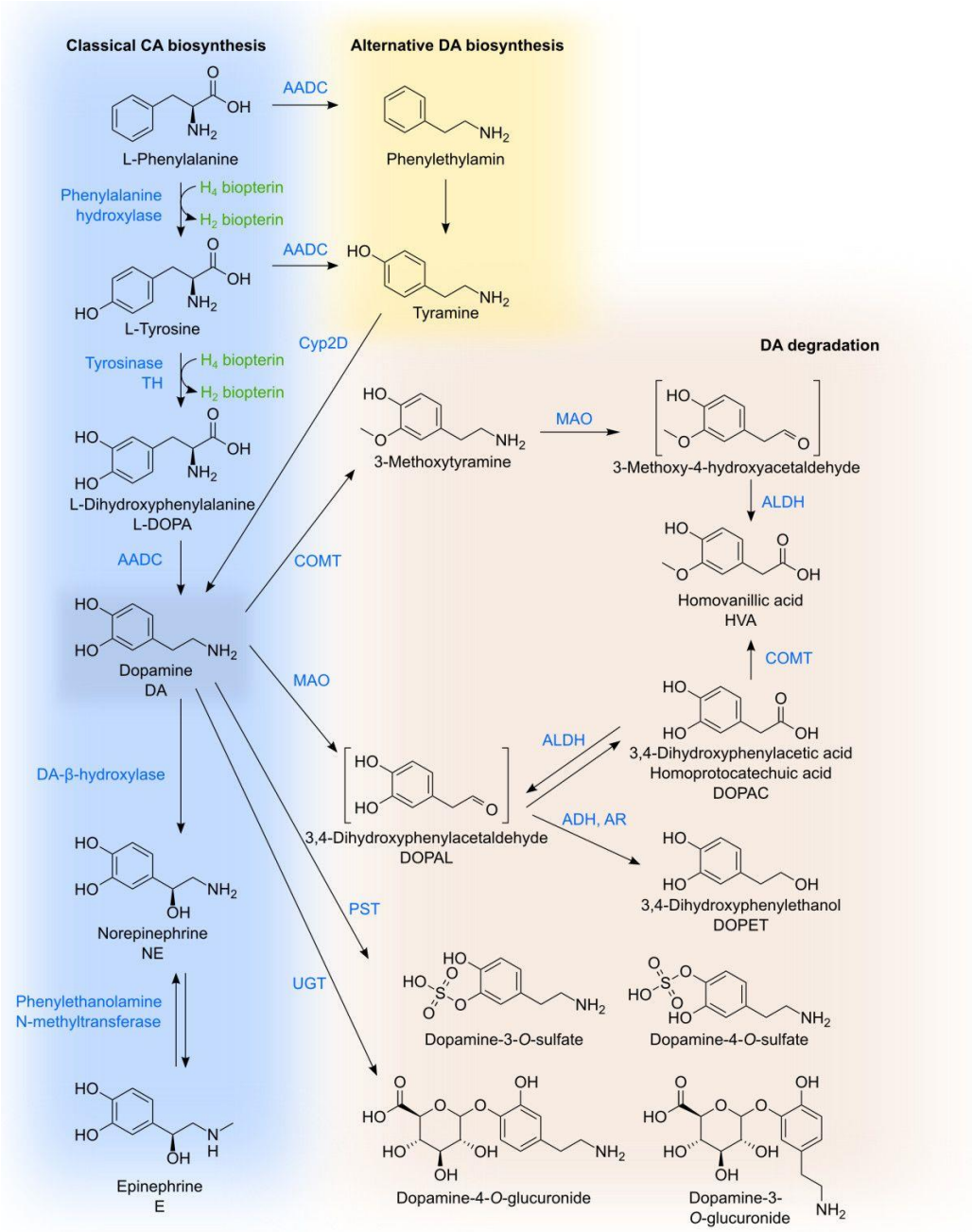

Figure 2 DA synthesis and metabolism. In the main synthesis pathway, L-phenylalanine is converted to L-tyrosine and further to levodopa by the rate limiting enzyme, TH. AADC converts levodopa to DA. DA is metabolized to 3-methoxytyramine by COMT and to DOPAL by MAO in the main metabolic pathways. 3-methoxytyramine is degraded further to HVA by MAO and ALDH. DOPAL is metabolized to DOPAC by ALDH or to DOPET by ADH. The main metabolites, DOPAC and HVA, undergo glucuronidation or sulphatation reaction, but DA can also be directly glucuronized or sulphatized. In noradrenergic and adrenergic cells, DA can be converted to norepinephrine and further to epinephrine. Adapted by permission from BioMed Central Ltd: Complexity of dopamine metabolism, Meiser J., Weindl D., Hiller K. Vol 11, copyright (2013).

In the brain, MAO is localized in neurons, astrocytes, and glial cells, but presence of MAO is low in the nigrostriatal neurons compared to other neurons (Westlund et al. 1988). MAO has two isoforms: MAO-A and MAO-B

(Bach et al. 1988, Johnston 1968). Both isoforms are localized on the outer mitochondrial membrane, but they have differences in affinity to DA in vivo; in humans DA is oxidized mainly by MAO-B but in rats by MAO-A (Napolitano et al. 1995). COMT is present only in the glial cells in the nigrostriatal tract (Myöhänen et al. 2010). DA synthesis and metabolism pathways are presented in Figure 2 and neuronal localization of synthesis and metabolism phases is presented in Figure 3.

2.1.2 DOPAMINE STORAGE AND RELEASE

Presynaptic vesicles are formed from plasma membrane by endocytosis. After the DA synthesis or reuptake of DA, VMAT2 transfers DA from cytosol into synaptic vesicles where it is stored until action potential signal induces fusion of the vesicles to the presynaptic membrane releasing DA to the synaptic cleft by exocytosis (Fon *et al.* 1997, Guillot & Miller 2009) (Figure 3). Influx of extracellular calcium (Ca^{2+}) causes fast release of neurotransmitters in less than 1 ms after the Ca^{2+} influx (Jahn & Scheller 2006, Martens & McMahon 2008). DA containing vesicles are transferred to the plasma membrane by motor proteins and vesicles interact with soluble N-ethylmaleimide-sensitive factor-attached receptor (SNARE) complex proteins that dock and then prime vesicles for membrane fusion (Li & Chin 2003).

2.1.2.1 Vesicular monoamine transporter (VMAT)

The main function for VMATs is packaging DA and other neurotransmitters from cytoplasm into synaptic vesicles. They are members of the solute carrier (SLC) protein family. The VMAT2 isoform is expressed mostly in neuronal cells in the brain, while isoform VMAT1 is expressed in neuroendocrine cells in the periphery (Erickson *et al.* 1996, Henry *et al.* 1994). VMAT2 is localized principally in small vesicles close to presynaptic membranes in the striatal axons and nerve terminals (Nirenberg *et al.* 1995, Nirenberg *et al.* 1997b, Nirenberg *et al.* 1997a). VMAT2 is a stoichiometric antitransporter that transfers two H^+ ions out of the vesicle while transporting one monoamine molecule into the vesicle (Knoth *et al.* 1981, Johnson *et al.* 1981). In the DAergic cells, VMAT2 regulates neurotransmission but also protects cells from the toxicity of DA and its metabolites since DA is not degraded in the acidic lumen of the vesicle as easily as in the non-acidic environment in the cytosol (Caudle *et al.* 2007, Guillot & Miller 2009, Kariya *et al.* 2005). VMAT2 is able to modulate DA release by controlling DA concentration in the synaptic vesicles, and thus is regulating the amount of DA released in the synapse (Colliver *et al.* 2000). Increase in cytosolic DA level or increase in VMAT2 expression are both able to increase the DA release by elevating the DA concentration in the vesicles (Pothos *et al.* 1996, Pothos *et al.* 2000).

Activation of DA receptor D2 increases VMAT2 function and induces redistribution of VMAT2 in striatal synaptosomes (Truong *et al.* 2004).

2.1.2.2 Soluble N-ethylmaleimide-sensitive factor-attached receptor (SNARE) complex

SNARE proteins compose a fundamental proportion of more than 50 proteins that are involved in neurotransmitter release. SNARE proteins are classified into two groups: vesicle-SNAREs that are bound to synaptic vesicles, i.e. vesicle-associated membrane protein 2 (VAMP2) also known as synaptobrevin, and the target-SNAREs, i.e. synaptosomal nerve-associated protein 25 kDa (SNAP-25) and syntaxin-1, that are enriched in lipid rafts and bound to the presynaptic membrane (Chamberlain *et al.* 2001, Wickner & Schekman 2008). VAMP2, SNAP-25, and syntaxin-1 are essential for normal neurotransmitter release (Jahn & Scheller 2006, Jones *et al.* 2001, Cervinski *et al.* 2010, Schoch *et al.* 2001, Sørensen *et al.* 2003). Assembly of vesicle-SNAREs and target-SNAREs leads to formation of a highly stable four-helix bundle called the SNARE complex which brings synaptic vesicle membrane and presynaptic membrane to close proximity and induces fusion of the membranes leading to DA release to the synaptic cleft by exocytosis (Söllner *et al.* 1993).

DA is able to promote SNARE-complex formation (Fisher & Braun 2000). Protein kinase C (PKC) has been suggested to regulate DA storage and release since PKC activator PMA increases plasma membrane localization of DA containing vesicles and enhances depolarization-induced DA release in PC12 cells (Shoji-Kasai *et al.* 2002).

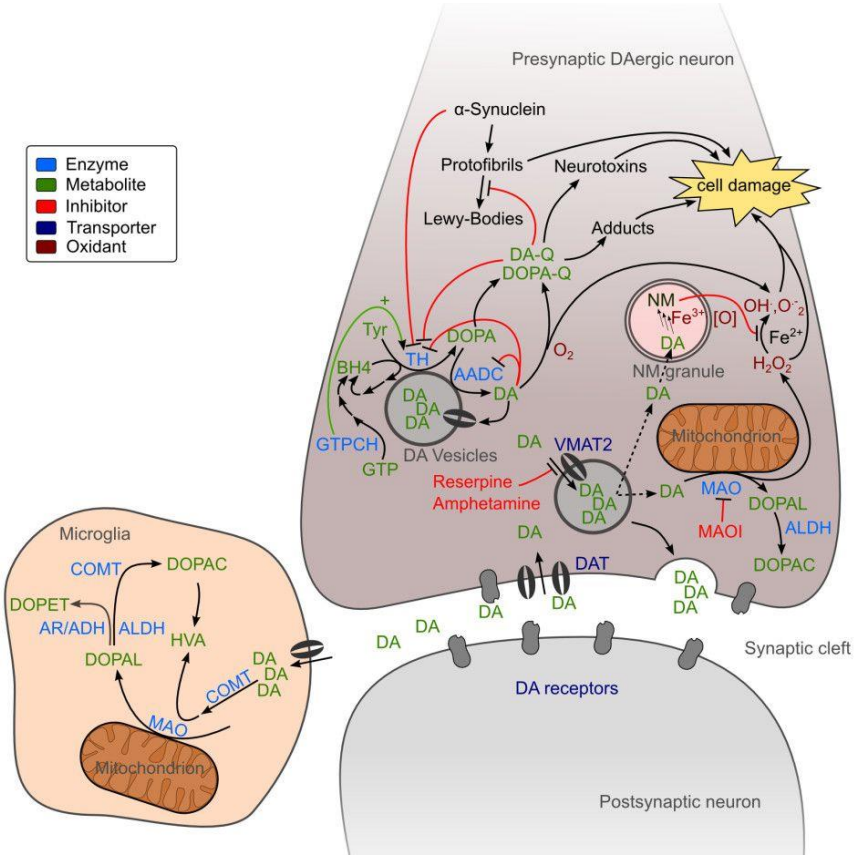

Figure 3 Neuronal DA metabolism. DA is synthesized in the presynaptic DAergic neuron and stored in the synaptic vesicles. DA is released from the vesicles by exocytosis to the synaptic cleft where it can interact with DA receptors. After the release, DA is removed from the synaptic cleft by diffusion and by active transportation by DAT. In the presynaptic cells, DA is recycled or degraded by MAO, and in the glial cells degraded by COMT and MAO. Adapted by permission from BioMed Central Ltd: Complexity of dopamine metabolism, Meiser J., Weindl D., Hiller K. Vol 11, copyright (2013).

2.1.3 DOPAMINE TRANSPORTER

DAT is a highly conserved presynaptic membrane protein (Amara *et al.* 1997, Nirenberg *et al.* 1996) that is essential in terminating DAergic signaling by removing DA from the synaptic cleft to the presynaptic neuron. DA is removed from the synaptic cleft mostly by diffusion after DA release, but DAT is the main regulator of the kinetics of extracellular DA (Rice & Cragg 2008). DAT belongs to the SLC6 transporter family of sodium/chloride (Na^+/Cl^-)-dependent neurotransmitter transporters (Ramamoorthy *et al.* 2011). It is composed of 12 transmembrane segments that form the core of the substrate translocation pathway and large N- and C-terminal domains that are oriented toward the cytoplasm (Giros *et al.* 1992). These terminals are the primary regions for post-translational modifications and for protein-protein interactions regulating DAT function. DAT transports DA against its concentration gradient by co-transport of Na^+ and Cl^- along their concentration gradients. DAT is primarily expressed on the plasma membrane where it can interact with extracellular DA.

DAT function is regulated by post-translational modifications, protein-protein interactions and internalization (German *et al.* 2015). Phosphorylation, ubiquination, glycosylation, palmitoylation, oxidation, and nitrosylation are able to modulate DAT function by changing DAT interactions with other molecules, regulating localization of DAT or altering DAT transport kinetics. Transport capacity of DAT can be modulated acutely as a physiological response to a variety of conditions and signaling, and dysfunction in these functions can have an important role in the pathogenesis of several neurological diseases (German et al. 2015, Foster & Vaughan 2017). Pharmacological modulation of post-translational modifications can provide numerous targets for treatment of DAT-related neurological diseases.

There are several kinases and other molecules that have long term effects on DAT function. The most studied kinases regulating DAT function are protein kinase C (PKC) (Vaughan *et al.* 1997), calcium-calmodulin dependent kinase II (CAMKII) (Fog *et al.* 2006), and extracellular signal-regulated protein (ERK) (Vaughan & Foster 2013, German et al. 2015, Foster & Vaughan 2017), but there are also some studies indicating a regulatory role for protein kinase A (PKA) (Batchelor & Schenk 1998), mitogen-activated protein kinase (MAPK) (Morón *et al.* 2003), tyrosine kinases (Hoover *et al.* 2007), arachidonic acid (Chen *et al.* 2003), Akt (Speed *et al.* 2010), phosphatidyl inositol 3-kinase (Carvelli *et al.* 2002), nitric oxide (Pogun *et al.* 1994), protein phosphatase 2A (PP2A) (Yang *et al.* 2018). Additionally, DA receptors D2 (Lee *et al.* 2007), and D3 (Zapata *et al.* 2007) can act as autoreceptors and regulate DAT function. Also, psychostimulants are able to modulate DAT function by several mechanisms.

Internalization is the main regulatory mechanism for DAT transport capacity, and phosphorylation of DAT regulates internalization (Melikian & Buckley 1999, Vaughan et al. 1997, Daniels & Amara 1999). Phosphorylation sites are located in the intracellular N- and C-terminals of DAT. Phosphorylation of Thr53 site in the MAPK/SH3 domain stabilizes DAT on the plasma membrane (Morón et al. 2003) while phosphorylation of Ser7 and Ser13 in the PKC domain induce internalization of DAT (Vaughan et al. 1997, Foster & Vaughan 2017) (Figure 4).

2.1.3.1 Protein kinase C

PKC-mediated phosphorylation of DAT has been suggested as one of the main regulators for DAT function (Vaughan et al. 1997). It was first shown that PKC activators can decrease DA uptake in striatal synaptosomes (Copeland *et al.* 1996). PKC induces phosphorylation of Ser7 and Ser13 in the PKC domain in the N-terminal of DAT leading to internalization of phosphorylated DAT (Loder & Melikian 2003, Vaughan et al. 1997, Pristupa *et al.* 1998, Karam *et al.* 2017). Internalization decreases DAT on the plasma membrane and leads to downregulation of the transport capacity. PKC also activates ERK, and thus is also able to modulate MAPK/SH3 domain phosphorylation.

2.1.3.2 Extracellular signal-regulated kinase

ERK is a negative regulator for DA signaling (Bolan *et al.* 2007). Phosphorylation activates ERK and leads to phosphorylation of the Thr53 site in the MAPK/SH3 domain located in the N-terminal of DAT (Morón et al. 2003, Challasivakanaka *et al.* 2017, Foster *et al.* 2012). This induces upregulation of DAT on the plasma membrane and increases transport capacity leading to decreased extracellular DA. Amphetamines elevate ERK activity, and ERK inhibition is able to block their effect showing that their effect is dependent on ERK-mediated phosphorylation of DAT (Foster et al. 2012, Shi & McGinty 2006).

2.1.3.3 Calcium-Calmodulin dependent kinase II

CaMKII is a cellular target for Ca^{2+} which colocalizes and interacts with DAT (Fog et al. 2006, Steinkellner *et al.* 2012). CaMKII phosphorylates serines in the C-terminal and induces DA efflux. Amphetamine-induced DA efflux is also dependent on CaMKII (Fog et al. 2006, Steinkellner *et al.* 2014, Steinkellner et al. 2012).

2.1.3.4 Psychostimulants

Psychostimulants, such as methamphetamine, amphetamine and cocaine, are also able to modulate DAT function, and they have been widely used for preclinical research of DAergic function. Methamphetamine and amphetamine regulate localization of DAT and they are also able to reverse DA transport from the cytosol (Sulzer 2011, Gulley & Zahniser 2003). Acute administration of cocaine blocks DAT transport, while chronic administration upregulates DAT function (Volkow *et al.* 1997, Mash *et al.* 2002). Amphetamines induce internalization of DAT even though they phosphorylate the Thr53 site in the N-terminal, while cocaine does not have a similar mechanism (Cervinski *et al.* 2005, Saunders et al. 2000, Challasivakanaka et al. 2017). Amphetamine- and methamphetamine-mediated phosphorylation and internalization are dependent on PKC activity (Cervinski *et al.* 2005, Richards & Zahniser 2009). Amphetamines also increase cytosolic DA by activating TH, inhibiting MAO, and impairing VMAT2 function (Sulzer 2011).

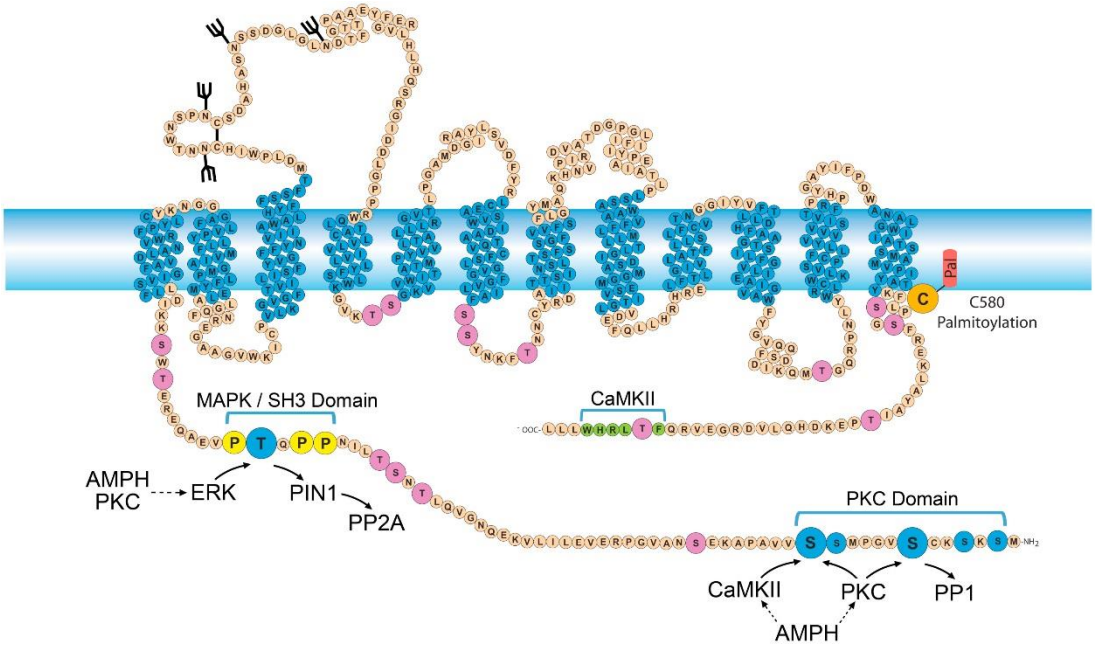

Figure 4 Phosphorylation of DAT. DAT has 12 transmembrane helices that form the core of the substrate translocation pathway, and large C- and N-terminals pointing toward the cytoplasm. The most important N-terminal located phosphorylation sites Thr53 in MAPK/SH3 binding domain, and Ser7 and Ser13 in PKC binding domain are marked with large blue circles. C-terminal located CaMKII binding domain is marked with green circles. Journal of chemical neuroanatomy, Vol 83, Foster, J. D. and Vaughan, R. A., Phosphorylation mechanisms in dopamine transporter regulation., pages 10-18., copyright (2017), with permission from Elsevier.

2.1.4 DOPAMINE RECEPTORS

After release, DA can bind to DA receptors located on the presynaptic or postsynaptic membrane. DA receptors are categorized as D1 like receptors, which includes receptors D1 and D5, and D2 like receptors, which includes receptors D2, D3 and D4 (Baik 2013, Klein *et al.* 2018). All DA receptors are metabotropic that act through a second messenger that modulates specific signaling pathways. D1 like receptors are located in the postsynaptic membranes mostly in the STR, SN, nucleus accumbens, olfactory bulb, amygdala, and frontal cortex (Savasta *et al.* 1986, Baik 2013, Wamsley *et al.* 1989). D2 and D3 receptors are located in both presynaptic and postsynaptic membranes, and found mainly in the STR, globus pallidus, nucleus accumbens, ventral tegmental area, hypothalamus, amygdala, cortical areas, hippocampus, and pituitary (Wamsley *et al.* 1989, Yokoyama *et al.* 1994, Baik 2013). D4 receptors are expressed mostly in retina (Cohen *et al.* 1992). D1 like receptors have lower affinity to DA than D2 like receptors (Baik 2013, Klein *et al.* 2018). D1 like receptors are suggested to be preferentially activated by high concentrations of DA phasic release, while D2 like receptors would detect tonic low levels of DA.

Presynaptically located DA receptor D2 and D3 act as autoreceptors regulating DA release (Beaulieu & Gainetdinov 2011). Activation of

presynaptic DA receptor D2 decreases DA release. DA receptor D2 promotes plasma membrane localization of DAT via direct protein-protein interaction leading to enhanced DA uptake (Lee *et al.* 2007) but DA receptor D2 also regulates DAT function via ERK1/2 (Bolan *et al.* 2007). Disruption of DAT and DA receptor D2 interaction has a neuroprotective effect (Su & Liu 2017). DA receptor D3 is also able to modify DAT function by activating phosphatidyl inositol 3 and MAPK (Zapata *et al.* 2007, Zapata & Shippenberg 2002).

2.2 OVERVIEW OF α-SYNUCLEIN

aSyn is a small, 140 amino acid protein encoded by SNCA gene, and is a member of synuclein family that includes also β-synuclein and γ-synuclein (Clayton & George 1998, Polymeropoulos *et al.* 1997). aSyn is widely expressed in the brain but is highly concentrated into the presynaptic nerve terminals (Spillantini *et al.* 1997, Iwai *et al.* 1995), and there is high expression levels in the DAergic brain regions (Taguchi *et al.* 2016). Survival and relatively normal development of aSyn knock-out mice has shown that aSyn is not essential (Chandra *et al.* 2004), but its physiological role is poorly understood, though modulation of DAergic neurotransmission has been suggested as its main function. aSyn has several protein-protein interactions and protein-lipid membrane interactions that are important for synaptic function homeostasis. It has been shown to regulate several steps of DAergic neurotransmission that are discussed later in this review. aSyn also interacts with a variety of cytoskeletal proteins that participate in the maintenance of cell structure and protein trafficking (Prots *et al.* 2013, Alim *et al.* 2002, Longhena *et al.* 2019). aSyn is able to modulate stability and organization of microtubules (Cartelli *et al.* 2016, Cartelli & Cappelletti 2017) and it affects anterograde axonal transport by binding to several proteins including tubulin, microtubule-associated protein 2, and tau (Prots *et al.* 2013, Longhena *et al.* 2019).

aSyn is mainly in an unfolded monomeric form in native conditions (Eliezer *et al.* 2001), but adopts α-helical conformation on membranes (Davidson *et al.* 1998, Chandra *et al.* 2003). Monomer and tetramer conformations are suggested to be the physiological forms of aSyn, while oligomers and fibrils are supposed as pathogenic forms (Bartels *et al.* 2011, Marques & Outeiro 2012). The soluble monomeric, unfolded aSyn and multimeric membrane-bound forms are suggested to have equilibrium that depends on N-terminal acetylation (Neupane *et al.* 2014, Bartels *et al.* 2010).

2.3 DOPAMINE AND α-SYNUCLEIN IN PARKINSON´S DISEASE

Parkinson´s disease (PD) is the second most common neurodegenerative disorder originally described by James Parkinson in 1817 (Parkinson 1817).

The motor symptoms tremor, rigidity, and slowness of movements are caused by loss of DAergic neurons in the nigrostriatal pathway in the brain (Ehringer & Hornykiewicz 1960). Parkinson´s disease is usually diagnosed when approximately 30 % of DAergic neurons in the SN and 50-60 % of their axon terminals in the STR are lost (Cheng *et al.* 2010). Neurodegeneration has been suggested to start from striatal nerve terminals then progressing later to the cell somas in the SN, which can explain wider loss of neurons in the STR compared to SN (Hornykiewicz 1998, Bridi & Hirth 2018). Also, reduction of immunoreactive DAT has been observed in the STR in PD brains (Miller *et al.* 1997). A PET study in early PD patients revealed 36% to 70% decreased binding potential of DAT in the presynaptic terminals in the STR and 30 % reduction in the cell bodies in the SN (Fazio *et al.* 2018).

The reason for DAergic neurodegeneration has remained obscure but multiple studies have revealed contribution of oxidative stress in the cell loss (Puspita *et al.* 2017). DA is a highly reactive molecule that can generate reactive oxygen species (ROS) such as hydroxyl radical, DA-o-quinone, superoxide, and hydrogen peroxide (Graham 1978). ROS are able to react with proteins, DNA and lipids, and these oxidatively modified molecules are found in the brain of PD patients (Spencer *et al.* 2002, Good *et al.* 1998, Alam *et al.* 1997). The highly reactive DA metabolite, DOPAL, is even more prone to induce oxidative stress than DA (Kristal *et al.* 2001) and it is toxic to neurons *in vitro* (Goldstein *et al.* 2012) and *in vivo* (Burke *et al.* 2003). DOPAL is increased in the PD brain which can be observed as elevated DOPAL:DA and DOPAL:DOPAC ratios (Goldstein *et al.* 2011). This elevation is caused by combination of decreased ALDH activity and decreased vesicular uptake of cytosolic DA by VMAT2 (Goldstein *et al.* 2013, Pifl *et al.* 2014). Loss of DAergic cells reduces DA related proteins VMAT2 and DAT in the PD brain, but DA uptake by VMAT2 is also reduced after correcting for DA nerve terminal loss (Pifl *et al.* 2014, Miller *et al.* 1997). Neuroimaging of VMAT2, DAT, and AADC with positron emission tomography (PET) or single photon emission computed tomography (SPECT) can be used in clinical research of PD (Kaasinen & Vahlberg 2017). Reduced VMAT2 has also been observed in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-model of PD in non-human primates (Chen *et al.* 2008). Intracerebral injection of DOPAL to rodents produces behavioral changes and pathology similar to PD showing that administration of DOPAL can be used to model PD and indicating that elevated DOPAL can lead to increased oxidative stress, toxicity, and DAergic cell loss in PD (Panneton *et al.* 2010, Burke *et al.* 2003).

In addition to DAergic dysfunction and cell loss another main pathological finding in the PD brain is protein aggregates called Lewy bodies that are mainly composed of aSyn (Spillantini *et al.* 1997). PD is commonly idiopathic, but there are also familial forms of PD. Mutations in various genes have been linked to PD including SNCA gene coding aSyn (Singleton *et al.* 2003, Polymeropoulos *et al.* 1997, Krüger *et al.* 1998). The most common point mutations in the SNCA gene are alanine to threonine substitution at position

53 (A53T), and alanine to proline substitution at position 30 (A30P) (Krüger *et al.* 1998, Polymeropoulos *et al.* 1997). These mutations have been shown to accelerate oligomerization and aggregation of aSyn (Conway *et al.* 2000, Conway *et al.* 1998). Moreover, duplication or triplication of SNCA causes familial PD (Chartier-Harlin *et al.* 2004, Singleton *et al.* 2003). Also, an A53E-associated mutation of SNCA gene has been found, which is linked to familial PD from Finland (Pasanen *et al.* 2014, Pasanen *et al.* 2017). Additionally, mutations in PTEN-induced putative kinase 1 (PINK1), Parkin, DJ-1, and leucine-rich repeat kinase 2 (LRRK2), among others, are associated to PD (Hernandez *et al.* 2016) but they are not discussed further in this thesis. Mutated aSyn or overexpression of aSyn have been widely used to model PD in cell cultures and in animals.

Phosphorylation of serine 129 residue promotes fibrillization of aSyn *in vitro* and this residue is selectively and extensively phosphorylated in synucleinopathy lesions (Fujiwara *et al.* 2002). Increase in ser-129 phosphorylated aSyn has been found in the cerebrospinal fluid of PD patients when total aSyn remained unaltered (Wang *et al.* 2012). However, phosphorylation of aSyn has been shown to decrease neurotoxicity in *Drosophila* (Chen & Feany 2005) and in rats (Gorbatyuk *et al.* 2008). Ser-192 phosphorylation also induces degradation of aSyn via autophagic and proteasomal pathways (Machiya *et al.* 2010).

Current treatments for PD are focused on increasing DA levels in the brain or to improve DAergic signaling. The precursor for DA, levodopa, is the golden standard in the treatment of motor symptoms of PD (Kalia & Lang 2015). It can alleviate symptoms efficiently by elevating DA level in the DAergic neurons of the central nervous system, but the efficiency of levodopa treatment reduces over time, and levodopa can cause severe side effects such as fluctuations and dyskinesia. Levodopa has a short biological half-life but COMT inhibitors, such as entacapone and opicapone, are used to inhibit DA metabolism and to extend the effect of levodopa. Additionally, MAO-B inhibitors, such as selegiline, rasagiline, and safinamide, are used to reinforce the effect of levodopa since they reduce DA metabolism and re-uptake, and inhibit presynaptic DA receptors. DA agonists, such as pramipexole, ropinirole, and rotigotine, can reduce motor symptoms by stimulating D2 and D3 receptors. Current medications can alleviate the PD symptoms, but they are not able to delay or stop the progression of the disease, thus raising urgent need for better understanding of the disease mechanisms and development of new treatments to modulate the progression of the disease.

2.4 A-SYNUCLEIN AS MODULATOR OF DOPAMINERGIC NEUROTRANSMISSION

aSyn participates in modulation of DAergic neurotransmission by interacting with several regulators of DA synthesis, metabolism, storage, release, and

clearance. Many of these interactions are altered in PD but their importance in the pathogenesis of PD has remained obscure. These interactions are discussed in detail below and they are gathered in Table 1.

2.4.1 DIRECT INTERACTION BETWEEN α-SYNUCLEIN AND DOPAMINE

DA is toxic to cultured midbrain neurons but lack of aSyn protects neurons from toxicity and cell loss pointing to an interaction between DA and aSyn (Mosharov *et al.* 2009). DA interacts with aSyn and induces formation and accumulation of soluble SDS-resistant aSyn oligomers and inhibits conversion of oligomeric aSyn to fibrils *in vitro* (Cappai *et al.* 2005, Rochet *et al.* 2004, Conway *et al.* 2001) and in SH-SY5Y cells (Mazzulli *et al.* 2006). Oxidation of DA is required for the interaction in the cytosol (Mazzulli *et al.* 2007), but DA is quickly oxidized to reactive quinone species, hydrogen peroxide, and other electrophiles in the cytosolic pH (Graham 1978). Oxidized DA changes the conformation of aSyn leading to a conformation that is suggested to be a precursor for the oligomeric form of aSyn (Outeiro *et al.* 2009, Norris *et al.* 2005). Oligomerization of A53T and A30P mutated aSyn is faster than wild-type aSyn and DA is able to stabilize mutated aSyn oligomers more than wild-type aSyn oligomers *in vitro* which can possibly explain the faster progression of PD in patients with aSyn mutations (Follmer *et al.* 2007, Conway *et al.* 1998). Overexpression of mutated A53T aSyn results in no loss of DAergic cells in a mouse model of PD (Giasson *et al.* 2002) but Mor *et al.* (2017) showed that A53T mutated aSyn overexpression combined with elevated DA level leads to accumulation of toxic aSyn oligomers and induced progressive nigrostriatal degeneration proving that DA and aSyn also interact *in vivo*. DA-induced oligomerization of aSyn can possibly provide a link between DA- and aSyn-mediated toxicities and explain the selective accumulation of Lewy bodies in the DAergic cells and loss of DAergic neurons in PD.

2.4.2 α-SYNUCLEIN AND DOPAMINE SYNTHESIS

TH, the rate limiting enzyme in DA synthesis, is decreased in the nigrostriatal pathway in PD mainly due to loss of TH-positive DAergic neurons, but TH might also be decreased in the remaining neurons (Nagatsu & Nagatsu 2016). Furthermore, overexpression of wild-type or mutated aSyn induces degeneration of TH-positive DAergic neurons in animal models of PD (Kirik *et al.* 2002, Kirik *et al.* 2003). Perez *et al.* (2002) showed that aSyn is able to bind to TH and inhibit TH activity *in vitro*. Overexpression of aSyn did not have an effect on TH protein level but decreased activity of TH leading to decreased DA synthesis (Perez *et al.* 2002). Silencing of aSyn has an opposite effect by increasing TH activity and DA synthesis in DAergic MN9D cells (Liu

et al. 2008). Additionally, the amount of immunoreactive TH was elevated in aSyn/γ-synuclein double knock-out mouse retinal cells (Surgucheva *et al.* 2005), although aggregated aSyn is not able to modulate TH activity (Alerte *et al.* 2008). In addition to direct binding, aSyn regulates TH activity by activating protein phosphatase 2A (PP2A) which reduces phosphorylation and activity of TH (Hua *et al.* 2015, Peng *et al.* 2005). Phosphorylation of aSyn reduces its impact on TH and PP2A activity and thus upregulates TH activity and DA synthesis (Wu *et al.* 2011, Lou *et al.* 2010). In PD, increase in aggregated aSyn and decrease in soluble aSyn could increase TH activity leading to increased DA synthesis and cellular DA concentration which can possibly cause oxidative stress and damage to the cells.

aSyn also interacts with another substantial enzyme in DA synthesis, AADC which converts levodopa to DA, by inhibiting its activity in a DAergic cell line (Tehranian *et al.* 2006). However, the interaction between aSyn and AADC and its relevance in DA synthesis and PD has not been studied as extensively as the interaction between TH and aSyn.

2.4.3 α-SYNUCLEIN AND DOPAMINE RELEASE AND STORAGE

aSyn binds to small phospholipid vesicles containing acidic phospholipids but not to vesicles with neutral charge or large vesicles *in vitro* (Davidson *et al.* 1998). Oligomeric aSyn has been shown to permeabilize vesicles *in vitro* indicating that oligomeric aSyn could also damage DA containing vesicles in the DAergic nerve terminals and cause leaking of DA to the cytosol (Volles *et al.* 2001). Membrane bound aSyn is preferentially in an α-helical form but oligomeric aSyn has a predominantly β-sheet structure (Davidson *et al.* 1998, Volles *et al.* 2001). aSyn is located in the presynaptic terminals close to synaptic vesicles and it has been shown to have interactions with cellular membranes *in vivo* (Kahle *et al.* 2000, Jo *et al.* 2000, Jo *et al.* 2002). Wild-type aSyn binds to lipid rafts on cellular membranes but the A30P mutation disrupts the interaction with lipid rafts and leads to decreased accumulation of aSyn in the synaptic terminals (Fortin *et al.* 2004). Absence of aSyn leads to decreased reserve pool of presynaptic vesicles and slower replenishment of the docked vesicles indicating that aSyn is needed for normal synaptic function (Cabin *et al.* 2002). There have also been studies showing that overexpression of wild-type or A30P mutated aSyn inhibits catecholamine release without affecting calcium threshold, vesicular pool or packing of the vesicles (Larsen *et al.* 2006). However, overexpression of aSyn reduced the readily releasable pool of vesicles.

Abeliovich *et al.* (2000) showed that aSyn deficient mice have intact morphology of the brain and nigrostriatal pathway including DA neurons and nerve terminals but the DAergic neurotransmission is altered. DA release and reuptake are normal, but recovery of DA release is faster after paired-pulse stimulation, DA tissue concentration is lowered and response to amphetamine altered indicating that aSyn is able to decrease vesicular storage and is acting

as an activity-dependent negative regulator of DA release. Lack of synucleins also impairs synaptic vesicle endocytosis, and restoration of mouse aSyn expression is able to normalize endocytosis in the mouse brain indicating that aSyn is needed for normal synaptic vesicle endocytosis function (Vargas *et al.* 2014).

Overexpression of mutated A30P or A53T aSyn has been shown to elevate cytosolic catecholamine concentration in PC12 cells suggesting increased leakage of neurotransmitters from storage vesicles (Mosharov *et al.* 2006). Mice lacking aSyn or overexpressing A30P mutated aSyn have a lower capacity of the DA storage pool (Yavich *et al.* 2004). (Chadchankar *et al.* 2011) had similar findings of altered DA release in aSyn deficient mice and they also found altered DA reuptake. Additionally, overabundant human wild-type aSyn is able to decrease neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis (Nemani *et al.* 2010, Gaugler *et al.* 2012). The mechanism in aSyn-mediated modulation of synaptic vesicle recycling is suggested to be inhibition of phospholipase D2 (PLD2) which leads to reduced formation of the vesicles (Jenco *et al.* 1998, Ahn *et al.* 2002). Mutated A53T aSyn has a greater ability to inhibit PLD2 than wild-type aSyn (Payton *et al.* 2004). Lipid-binding is also suggested to stabilize aSyn into its active α-helical conformation (Payton *et al.* 2004).

aSyn localizes with VMAT2 in Lewy bodies in PD brains (Yamamoto *et al.* 2006). aSyn forms complexes with VMAT2 and overexpression of aSyn decreases VMAT2 expression in SH-SY5Y cells leading to decreased VMAT2 function and elevated intracellular ROS level (Guo *et al.* 2008). Overexpression of A53T mutated aSyn decreases VMAT2 protein level and VMAT2-mediated uptake of DA in cells (Lotharius & Brundin 2002). Overexpression of VMAT2 protects from levodopa-induced toxicity (Mosharov *et al.* 2009), while reduction of VMAT2 increases oxidative stress, DAergic neurodegeneration, and accumulation of aSyn in the SN of aged VMAT2 deficient mice (Caudle *et al.* 2007). Additionally, VMAT2 deficiency combined with aSyn overexpression increases cytosolic DA concentration and leads to DAergic neurodegeneration in mice (Ulusoy *et al.* 2012).

aSyn colocalizes with SNARE proteins, syntaxin-1 and SNAP-25, and age-dependent redistribution of SNARE proteins and reduction of DA release was observed in aSyn transgenic mice (Garcia-Reitböck *et al.* 2010, Almandoz-Gil *et al.* 2018). aSyn aggregates have been shown to reduce VAMP2 and SNAP-25 in cells and in mouse brain, while monomeric aSyn did not a have similar effect (Choi *et al.* 2018). There has also been opposite results showing that aSyn binding to VAMP2 promotes SNARE-complex assembly by increasing clustering of vesicles but overexpression of aSyn still decreased the releasable pool (Burré *et al.* 2010, Diao *et al.* 2013). α-, β- and γ-synuclein triple knock-out mice have been shown to develop age-dependent neurological impairment and decreased SNARE-complex assembly indicating that synucleins have an important role in the regulation of SNARE-complex assembly during aging. Monomeric cytosolic aSyn does not promote SNARE-complex assembly but

the multimeric membrane-bound form of aSyn is able to mediate SNARE-complex assembly in the presynaptic nerve terminals (Choi *et al.* 2013, Burré *et al.* 2014). Large aSyn oligomers have the opposite effect as they inhibit SNARE-mediated lipid mixing by preventing SNARE-complex formation (Choi *et al.* 2013). aSyn concentration might also affect the interaction with SNARE-complex since low concentrations (<2.5 μM) promoted vesicle docking and high concentrations (>4 μM) inhibited docking in the study by (Lou *et al.* 2017). A suggested mechanism for promotion of SNARE-complex assembly is aSyn interaction with proteoliposomes in both synaptic and vesicle SNARE which could cross-bridge the membranes and promote their fusion (Lou *et al.* 2017). According to variable results, the effect of aSyn on the SNARE-complex is dependent on the conformation and concentration of aSyn. Furthermore, inhibition of SNARE-complex assembly by aSyn oligomers could possibly explain the toxicity of the oligomers.

2.4.4 α-SYNUCLEIN AND DOPAMINE METABOLITES

DOPAC, one of the main metabolites of DA, is able to bind to aSyn at low concentrations and prevent fibrillization of aSyn by stabilizing it on the monomeric form *in vitro* (Zhou *et al.* 2009). Interestingly, aSyn induces spontaneous oxidation of DOPAC, and the oxidation produces H_2O_2 which can cause oxidation of methionine groups of aSyn leading to formation of soluble oligomers and inhibition of fibrillization (Zhou *et al.* 2009). Additionally, the interaction between aSyn and DOPAC decreases binding affinity of aSyn to lipids suggesting that DOPAC can inhibit normal function of aSyn and increase the toxicity of aSyn aggregates (Zhou *et al.* 2010).

The toxic metabolite of DA, DOPAL, induces aggregation of aSyn both *in vitro* and *in vivo* (Burke *et al.* 2008) and aSyn oligomerization *in vitro* and in cells (Plotegher *et al.* 2017, Jinsmaa *et al.* 2016). DOPAL injection in the rat SN causes accumulation of high molecular weight oligomers of aSyn suggesting that DOPAL can cause the aSyn aggregation and DAergic cell loss observed in PD (Burke *et al.* 2008). Elevated DOPAL in the PD brain also supports this theory (Goldstein *et al.* 2013, Goldstein *et al.* 2011). DOPAL is shown to stabilize aSyn oligomers by formation of covalent adducts with lysine residues of aSyn (Follmer *et al.* 2015), and ROS produced by DOPAL are able to oxidize methionine residues of aSyn and therefore stabilize large aSyn oligomers (Follmer *et al.* 2015, Plotegher *et al.* 2017, Carmo-Gonçalves *et al.* 2018). Decrease in vesicular uptake of cytosolic DA and decrease in detoxification of DOPAL by ALDH are suggested to be the main reasons for elevated DOPAL in the PD brain (Goldstein *et al.* 2013). DOPAL-modified aSyn also damages cellular vesicles and DOPAL affects the synaptic vesicle pool (Plotegher *et al.* 2017). aSyn-DOPAL oligomers are able to permeabilize vesicles when membranes contain cholesterol *in vitro* suggesting that aSyn-DOPAL oligomers could possibly cause DA release from the vesicles to cytosol leading to oxidative stress and damage to the cells (Plotegher *et al.* 2017).

Overexpression of mutant A53T-aSyn causes transcriptional downregulation of COMT in transgenic mice leading to elevated striatal DA level but the downregulation of COMT might be a compensation for decreased extracellular DA level rather than direct interaction between aSyn and COMT (Kurz *et al.* 2010). Wild-type and mutated aSyn bind directly to another DA metabolizing enzyme, monoamine oxidase B (MAO-B), but not MAO-A, and aSyn stimulates MAO-B enzymatic activity (Kang *et al.* 2018) which could lead to increased accumulation of DOPAL and elevated oxidative stress.

2.4.5 α-SYNUCLEIN AND DOPAMINE TRANSPORTER

SiRNA knock-down of aSyn in a human neuronal cell line decreases DAT function and cell surface localization suggesting that endogenous aSyn promotes DAT activity and normal function of aSyn includes regulation of DAT activity (Fountaine & Wade-Martins 2007, Fountaine *et al.* 2008). However, altered DAT function has not been observed in aSyn knock-out mice (Chandra *et al.* 2004, Dauer *et al.* 2002). Impaired regulation of DAT by aSyn can lead to changes in extracellular and intracellular DA levels, and cellular damage and toxicity (Wersinger *et al.* 2003b). aSyn binds directly to DAT and forms a complex with DAT through binding of the non-Aβ component of aSyn and C-terminal tail of DAT (Lee *et al.* 2001, Wersinger & Sidhu 2003). DAT-aSyn complexes are located mainly on the plasma membrane indicating that aSyn is able to modulate DAT function by stabilizing it on the plasma membrane, and amphetamine is able to enhance the association between DAT and aSyn at the plasma membrane (Butler *et al.* 2015). aSyn-DAT complexes are also redistributed in the somatodendritic compartment in aSyn transgenic mice (Bellucci *et al.* 2011) suggesting aSyn-mediated modulation of somatodendritic DA release which can be substantial in motor functions (Hersch *et al.* 1997). Redistribution of aSyn-DAT complexes has also been observed in the caudate putamen of PD patients (Longhena *et al.* 2018). Both wild-type and mutated aSyn are able to form complexes with DAT but A53T has a weaker association with DAT than A30P or wild-type aSyn and it is not able to decrease DA-induced oxidative stress like A30P and wild-type aSyn (Wersinger *et al.* 2003a).

However, there are several studies reporting an aSyn-mediated decrease in DAT trafficking (Oaks *et al.* 2013, Wersinger & Sidhu 2003, Kisos *et al.* 2014, Wersinger *et al.* 2003b, Swant *et al.* 2011, Pelkonen *et al.* 2013) suggesting that aSyn is able to increase endocytosis of DAT by a chlatrin-dependent mechanism (Kisos *et al.* 2014) and to impair endoplasmic reticulum-Golgi transition leading to limited export of DAT from the endoplasmic reticulum (Oaks *et al.* 2013). A study by (Swant *et al.* 2011) suggests that aSyn can decrease DAT activity without changing the amount of DAT on the plasma membrane by altering the ionic coupling of DAT leading to decreased DA uptake. Wersinger & Sidhu (2003) showed that in its normal state, aSyn is a negative regulator for DAT function, and overexpression of aSyn can decrease

DAT function in an expression level-dependent manner but 1-Methyl-4-phenylpyridinium (MPP+)-induced toxicity ablated the aSyn-mediated inhibition of DAT activity in cells (Wersinger *et al.* 2003b). Overexpression of aSyn has shown to increase basal and amphetamine-induced DA efflux at resting membrane potential (Wersinger & Sidhu 2003). Controversial results suggest that the negative or positive impact on DAT trafficking by aSyn depends on the circumstances.

In addition to direct binding, aSyn can interact with DAT by tethering it to the microtubular network and decreasing cell surface-associated DAT, and microtubule destabilizing agents are able to reverse aSyn-mediated inhibition of DAT activity (Wersinger & Sidhu 2005). aSyn also binds to PKC and inhibits its activity (Ostrerova *et al.* 1999) suggesting that aSyn could decrease phosphorylation of DAT via PKC and thus enhance internalization of DAT (Huff *et al.* 1997, Vaughan & Foster 2013, Copeland *et al.* 1996). Another protein kinase, PKA, also regulates DAT function but aSyn does not have an effect on PKA (Peng *et al.* 2005). Since PP2A regulates DAT by modulating its phosphorylation (Yang *et al.* 2018, Bauman *et al.* 2000) aSyn could possibly regulate DAT function by increasing PP2A activity (Peng *et al.* 2005). DA receptor D2 is upregulated in the STR of aged A53T-aSyn overexpressing mice (Kurz *et al.* 2010) which may modulate DAT function as DA receptor D2 stabilizes DAT on the plasma membrane but DA receptor D2 upregulation is more likely a result of compensation to decreased DA level (Seeman & Niznik 1990) than direct interaction between aSyn and DA receptor D2.

Taken together, aSyn has interactions with several regulators of DAT function and it also directly binds to DAT suggesting many different mechanisms in aSyn-mediated regulation of DAT function. This can probably explain why aSyn has either increased or decreased DAT function in different studies. Additionally, there have been different circumstances such as aSyn amount and conformation, mutated or wild-type aSyn, and different cell lines and models *in vitro* and *in vivo*.

Table 1. *aSyn interactions in DAergic neurotransmission.*

Target	Consequence	Reference
DA	Increased aSyn oligomerization	(Conway *et al.* 2001)
TH	Decreased TH activity and DA synthesis	(Perez *et al.* 2002)
PP2A	Downregulation of TH activity and DA synthesis	(Hua *et al.* 2015, Peng *et al.* 2005)
VAMP2	Membrane-bound aSyn promotes SNARE-complex assembly	(Burré *et al.* 2010, Burré *et al.* 2014)
VAMP2	aSyn oligomers inhibit SNARE-mediated vesicle docking	(Choi *et al.* 2013)
SNAP-25, VAMP2	aSyn aggregates reduce SNAP-25 and VAMP2 impairing SNARE complex formation	(Choi *et al.* 2018)
SNARE proteoliposomes	Promotion of vesicle docking in SNARE-complex	(Lou *et al.* 2017)
VMAT2	Reduced activity	(Lotharius & Brundin 2002)
PLD2	Inhibition reduces synaptic storage vesicles	(Ahn *et al.* 2002, Jenco *et al.* 1998)
DAT C-terminal	DAT-aSyn complex stabilizes DAT on the plasma membrane leading to increased DA uptake	(Lee *et al.* 2001, Wersinger & Sidhu 2003)
PKC	Inhibition alters DAT phosphorylation	(Ostrerova *et al.* 1999)
ERK	Inhibition alters DAT phosphorylation	(Ostrerova *et al.* 1999)
COMT	Downregulation decreases DA metabolism	(Kurz *et al.* 2010)
DOPAC	Increased oxidation of DOPAC and accelerated formation of soluble aSyn oligomers	(Zhou *et al.* 2009, Zhou *et al.* 2010)
DOPAL	Stabilization of aSyn oligomers, damage to cellular vesicles	(Plotegher *et al.* 2017)
MAO-B	Increased activity	(Kang *et al.* 2018)

2.5 THE ROLE OF PROLYL OLIGOPEPTIDASE IN DOPAMINERGIC NEUROTRANSMISSION

PREP is a highly conserved serine protease that cleaves small peptides shorter than 30 amino acids (Venäläinen *et al.* 2004, Camargo *et al.* 1979). PREP has been shown to cleave several peptide hormones *in vitro*, such as substance P, thyrotropin-releasing hormone, arginine-vasopressin, bradykinin, and neurotensin that are related to neurodegenerative diseases (Myöhänen *et al.* 2009a), but the physiological role of PREP has remained unclear (Männistö *et al.* 2007, Männistö & García-Horsman 2017). PREP is widely distributed in different tissues in mammals but the highest activities have been measured in the brain (Fuse *et al.* 1990, Kato *et al.* 1980, Agirregoitia *et al.* 2007, Goossens *et al.* 1996). PREP has been found to localize in the GABAergic and cholinergic interneurons of the thalamus and cortex, and in the GABAergic and DAergic neurons in the nigrostriatal pathway (Myöhänen *et al.* 2008, Myöhänen *et al.* 2009b). At the cellular level, PREP is mainly in the cytosol, and to some extent in the nucleus and bound to the membrane (Schulz *et al.* 2005, Myöhänen *et al.* 2009a). However, since the impact of PREP on neuropeptides *in vivo* has not been very conclusive (Jalkanen *et al.* 2012), the physiological role of PREP has remained a mystery. More recent studies have also suggested non-hydrolytic functions for PREP and that protein-protein interactions might be more relevant than the hydrolytic function for the physiological role of PREP (Svarcbahs *et al.* 2019).

PREP activity is altered in the brain tissue of PD, Alzheimer´s disease, Lewy body dementia, and Huntington´s disease patients (Mantle *et al.* 1996). Although earlier reports indicated elevated PREP activity, changes in activities differ a lot between the studies, and therefore, the true nature of PREP activity in neurodegenerative diseases is inconclusive. However, PREP colocalizes with aSyn in the PD brain, and with tau aggregates and beta-amyloid plaques in Alzheimer´s disease brains (Hannula *et al.* 2013), and it has been shown that PREP binds directly to aSyn and increases its aggregation *in vitro* and in mouse models of PD (Lambeir 2011, Savolainen *et al.* 2014, Savolainen *et al.* 2015). PREP inhibitors can prevent aggregation and enhance the clearance of aggregates and oligomers in aSyn overexpressing cells and in aSyn transgenic mice (Myöhänen *et al.* 2012, Dokleja *et al.* 2014).

PREP inhibitors KYP-2047 and JTP-4819 have been shown to decrease the extracellular concentration of DA in rat brain (Jalkanen *et al.* 2012) and increase tissue concentration of DA and immunoreactive DAT in the STR of A30P point-mutated transgenic mice (Savolainen *et al.* 2014). However, the mechanisms behind PREP regulation of the DA system are unclear. One possibility is that DAT function is regulated by ERK (Morón *et al.* 2003) and PREP inhibitors KYP-2047 and JTP-4819 are able to modulate phosphorylation of ERK (Moreno-Baylach *et al.* 2011, Tenorio-Laranga *et al.* 2013), indicating that PREP could possibly regulate DAT function via ERK. Additionally, the interaction between PREP and aSyn could possibly modify

the interactions between aSyn and the nigrostriatal DAergic system, but PREP also has direct interactions with DAT and other players in DAergic neurotransmission which are investigated further in this thesis.

3 AIMS OF THE STUDY

PREP has been shown to participate in regulation of the brain DAergic system in mice and rats. Although PREP inhibitors have been suggested for treating PD and possibly also for other neurodegenerative diseases, the mechanisms of PREP interaction with the nigrostriatal DAergic system are not known. The aim of this study was to characterize the role of PREP and aSyn in DAergic neurotransmission in the nigrostriatal tract in PD models.

The effect of PREP and PREP inhibition and the interaction between PREP, aSyn and DAT were studied *in vitro* and *in vivo*. The specific aims of the studies were:

I : To characterize the effect of expression, inhibition, absence and overexpression of PREP on DAT function in transfected HEK-293 cells and on the nigrostriatal DAergic function in mice.

II : To investigate the effect of PREP inhibitor treatment on behavior and the DAergic system in the AAV-aSyn mouse model of PD.

III : To study if the toxicity of aSyn is dependent on the presence of PREP, and impact of this on the mouse DAergic system.

4 MATERIALS AND METHODS

4.1 DRUGS AND REAGENTS

Reagents used in experiments were purchased from Sigma-Aldrich if not otherwise specified. Ethanol was purchased from Altia (Helsinki, Finland). PREP inhibitor, 4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine (KYP-2047) was synthetized at the School of Pharmacy, University of Eastern Finland, as described previously by (Jarho *et al.* 2004).

4.2 PLASMIDS

pAAV1-EF1α-hPREP (#59967; Addgene, Cambridge, United States), pAAV1-EF1α-S554A-hPREP (S554A-PREP; #59968; Addgene), and pAAV EF1a V5-aSyn (WT) (#60057; Addgene), have been described previously (Savolainen *et al.* 2014). pAAV-EF1a control vector with 50 bp insert was created by annealing complementary oligonucleotides (10x annealing buffer: 100 nM Tris HCl, 500 mM NaCl, 10 mM EDTA). This insert was recombined into the KpnI-HF (R3142; NEB, Ipswich, United States) and EcoRV-HF (R3195; NEB) sites of pAAV-EF1a-PREP vector using an In-Fusion HD cloning kit (639645; Clontech, Mountain View, United States). Plasmid was transformed into Stbl3 One-Shot competent cells (#C737303, ThermoFisher Scientific). An insert containing clone was verified by sequencing. pcDNA3.1-hDAT (#32810; Addgene) was a gift from Susan Amara.

4.3 CELL EXPERIMENTS

4.3.1 CELL LINES AND TRANSFECTION

HEK-293 and stable PREPko cells generated in HEK-293 background were used in the study. PREPko cells were generated as described in publication IV. Wild-type HEK-293 cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) containing an additional 10 % (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, USA) and 1 % (v/v) penicillin-streptomycin (Lonza, Basel, Switzerland). PREPko cells were cultured in DMEM containing an additional 20 % (v/v) FBS (Invitrogen) and 1 % (v/v) penicillin-streptomycin (Lonza). Cell cultures were maintained at 37 °C in humidified air containing 5 % CO_2.

4.3.2 WESTERN BLOTTING

To study phosphorylation of DAT and ERK by Western blotting, wild-type HEK-293 and PREPko cells were seeded with a density 6 x 10^5 cells per well the day before transfection on 6-well plates. Cells were transfected with DAT by using Lipofectamine 3000 transfection kit (Thermo Fisher, IL, USA) according to manufacturer´s protocol 2 days prior to cell lysis. DAT transfected cells were starved for 2 hours in serum free media prior to treatment with vehicle (0.01 % dimethyl sulfoxide (DMSO) (v/v)), 1 µM KYP-2047, 1 µM PMA or 1 µM PMA + 1 µM KYP-2047 for 30 minutes. DAT transfected cells were treated with vehicle (0.01 % DMSO (v/v)), 1 µM KYP-2047, 1 µM Gö-6983, or 1 µM Gö-6983 + 1 µM KYP-2047 without prior starvation.

Cells were lysed on 150 µl ice-cold modified RIPA buffer (50 mM Tris HCl pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl) containing Halt Phosphatase Inhibitor (Product# 87786, Thermo Fisher Scientific) and Halt Protease Inhibitor cocktail (Product# 78430, Thermo Fisher Scientific). Cells were homogenized with an ultrasound sonicator (GM35-400, Rinco Ultrasonic, Switzerland). After centrifugation at 13 300 g and + 4 °C for 15 min, the supernatants were collected. Protein concentration was measured by the bicinchoninic acid (BCA) method (Product #23225, Thermo Fisher Scientific). 30 µg of protein was loaded onto a 12 % Mini-PROTEAN TGX precast gel (Product # 4561044, Bio-Rad, CA, USA) for SDS-PAGE. Gel was transferred to PVDF membrane (Trans-blot Turbo, Product# 1620175, Bio-Rad) by using Trans-blot Turbo Transfer System (Bio-Rad). The membranes were blocked by 5 % skim milk. The membranes were incubated with primary antibody overnight at + 4 °C and thereafter with secondary antibody for 2 hours at room temperature. Antibodies were diluted in 5 % skim milk in 0,1 % Tween-20 in Tris-buffered saline (TTBS). The following primary antibodies and dilutions were used: DAT, rabbit anti-DAT (#PA1-4656, Thermo Fisher Scientific, dilution 1:1000); rabbit anti-phospho (T53)-DAT (pDAT) (#ab183486, AbCam, UK, dilution 1:500); ERK, rabbit anti-ERK1+ERK2 (#ab17942, AbCam, dilution 1:1000), phospho ERK (pERK), mouse anti-ERK1+ERK2 (#ab50011, Abcam, dilution 1:5000) β-actin, rabbit anti-β-actin (#4967S, Signaling Technology, MA, USA, dilution 1:1000). Goat anti-rabbit HRP (#31460, Thermo Fisher Scientific) and goat anti-mouse HRP (#31430, Thermo Fisher Scientific) were used as secondary antibodies (dilution 1:2000). The images were captured with ChemiDoc MP chemiluminescence scanner (Bio-Rad). Three independent Western blotting experiments were performed. Optical density (OD) values of the Western blotting images were analyzed using ImageJ software (NIH, MD, USA). The OD values were normalized to the loading control (β-actin) ODs. pDAT/DAT ratio and pERK/ERK ratio were calculated from values normalized to β-actin and wild-type control was set as 100 %.

4.3.3 DOPAMINE UPTAKE ASSAY

Cells were seeded (1.2 x 10^5 cells per well or 1.5 x 10^5 cells per well) the day before transfection in 24-well plates (Falcon 3047) coated with poly-l-lysine (Sigma-Aldrich). Cells were transfected 2 days prior to the experiment with DAT in combination with mock plasmid, PREP or S554A-PREP using Lipofectamine 3000 (Thermo Fisher) transfection protocol. Media was removed and cells were washed once with 0.25 ml uptake buffer (5 mM Tris, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid, 5 mM glucose; pH 7.1). Cells were preincubated in uptake buffer for 15 min (vehicle) or in uptake buffer containing 1 µM KYP-2047, 1 µM PMA, 1 µM Gö-6983, 1 µM PMA + 1 µM KYP-2047 or 1 µM Gö-6983 + 1 µM KYP-2047 for 30 min in + 37 °C prior to 5 min incubation with of 0.25 ml 20 nM [^{3}H] DA (PerkinElmer, Woodbridge, ON, Canada) containing 2 µM or 10 µM unlabeled DA in + 37 °C. Cells treated with Gö-6983 were preincubated in media containing 1 µM Gö-6983 for 10 minutes before the other treatments. To observe phosphorylation of ERK, PMA treated cells and their control cells were starved in serum free media for 2 hours before the treatments. Wells were washed three times with ice-cold 0.32 M sucrose and lysed with 0.25 ml of 0.25 M NaOH. Optiphase SuperMix (Perkin Elmer, Turku, Finland) scintillant was added to the wells, and radioactivity was quantified using a scintillation counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer, Turku, Finland) after placing cross-talk minimizing inserts (1450-109, Perkin Elmer) into each well. Counts per well were measured for 5 min.

4.3.4 PKC ACTIVITY ASSAY

PKC activity was measured from wild-type HEK-293 cells with a PKC Kinase Activity Assay Kit (ab139437, Abcam) according to the manufacturer's protocol. Briefly, cells were plated on poly-l-lysine (Sigma-Aldrich) coated 12-well plates with a density of 2.5 x 10^5 per well. The following day the cells were treated with 0.1 % DMSO (vehicle), 10 µM KYP, 100 nM PMA (Sigma), 1 µM Gö-6983, 10 µM KYP + 100 nM PMA or 1 µM Gö-6983 + 100 nM PMA. When Gö-6983 was used in combination with PMA, it was added to the cells 10 min before PMA. The cells were treated for 30 min in + 37°C and the treatments were performed in serum free media. After the treatment, the cells were washed once with PBS and lysed with lysis buffer containing 50 mM KH_2PO_4, 1.5 mM $MgCl_2$, 10 mM NaCl and 1 mM EDTA (pH 7.4) on ice. The cells were scraped, collected to Eppendorf tubes, and centrifuged (18 000 g, 15 min, + 4 °C). Supernatants were collected and diluted 1:20 to kinase assay dilution buffer provided in the kit. The diluted samples were pipetted to the 96-well plate included in the kit and the following steps were done according to the manufacturer's instructions. The protein concentrations of the samples were measured with BCA and the PKC activities were normalized to the protein levels.

4.4 *IN VIVO* STUDIES

4.4.1 ANIMALS

PREP gene knock out mice (PREPko) (Deltagene Inc, CA, USA) and wild-type littermates were back crossed in C57Bl/6JRccHsd genetic background (Envigo, The Netherlands; 5–10 back crossings). Generation of PREPko mice used in these experiments has been described by (Di Daniel *et al.*, 2009), and behavioral phenotyping was done by (Höfling *et al.*, 2016).

Mice (7-9 weeks old; Envigo, The Netherlands) were housed under standard laboratory conditions (12 h light/dark cycle; room temperature + 23±2 °C; relative humidity: 50±15 %) in individually ventilated cages (Mouse IVC Green Line, Techniplast, Italy) with bedding (Aspen chips 5 x 5 x 1 mm, 4HP, Tapvei, Estonia), nesting material (aspen strips, PM90L, Tapvei), and aspen brick (100 x 20 x 20 mm, Tapvei). Mice had access to chow food (Teklad 2016, Envigo) and filtered and irradiated water *ad libitum*. After surgical procedures, animals were individually housed for the duration of the experiment. The experiments were carried out according to the European Communities Council Directive 86/609/EEC and were approved by the Finnish National Animal Experiment Board. Animal lisence number is ESAVI/441/04.10.07/2016.

4.4.2 VIRAL VECTORS

Adeno-associated viral vectors (AAV), AAV1-EF1α-hPREP (AAV-PREP), and AAV1-EF1α-eGFP (AAV-GFP), were obtained from the National Institute of Drug Abuse (Dr. Brandon Harvey, Intramural Research Program, Baltimore MD, USA).

AAV vectors driven by chicken β-actin promoter (CBA) were acquired from the Michael J. Fox Foundation. AAV2-CBA-alpha-synuclein (AAV-aSyn; 1.5x1013 vg/ml) and AAV2-CBA-eGFP (enhanced green fluorescent protein; AAV-GFP; 8.1x1012 vg/ml) were constructed, produced, and titered by Vector Core at the University of North Carolina (Chapel Hill, USA). pOTTC414 - pAAV1-EF1α-hPREP (AAV-PREP; Addgene plasmid #59967), pOTTC407 - AAV1-EF1α-GFP (AAV-GFP; Addgene plasmid #60058) and pOTTC293 - AAV-EF1a-V5-synuclein (WT; Addgene plasmid # 60057) was obtained from National Institute of Drug Abuse (Dr. Brandon Harvey, Intramural Research Program, Baltimore, MD, USA).

4.4.3 SURGICAL PROCEDURES

Mice were anesthetized with isoflurane (4 % induction, 1.5–2.0 % maintenance) and supranigral injection of AAV vector was given in a stereotaxic operation. To target the SN, viral vectors were given as a single

injection (volume 1 µL or 2 µL co-injection, rate 0.2 µL/min) into the left hemisphere, 3.1 mm anterior and 1.2 mm lateral to bregma, and 4.2 mm below the dura, stereotaxic coordinates according to (Paxinos G 1997). Characterization of the viral vectors showed protein expression 2 to 4 weeks after the microinjections in both SN and STR (data not shown).

Animals for microdialysis experiments underwent the same set of virus vector microinjections (described above). Guide cannula (AT4.9.iC, AgnTho´s, Sweden) for microdialysis was inserted into the STR at 0.6 mm anterior and 1.8 mm lateral to bregma, and 2.7 mm below the dura 13-14 weeks after the injection of viral vector. The cannula was fastened to the skull with dental cement (Aqualox, Voco, Cuxhaven, Germany) and two stainless steel screws (1.2 × 3 mm, DIN84, Helsingin Ruuvihankinta, Finland).

4.4.4 CYLINDER TEST

The cylinder test (height, 15 cm; diameter, 12 cm) was used to measure motor asymmetry in spontaneous forelimb use after unilateral microinjections. Baseline paw preference scores were acquired prior to stereotaxic surgery and every 2 to 3 weeks after the surgery. Shortly, each mouse was filmed for 5 min. However, if the number of individual rearing episodes that resulted in the mouse touching the cylinder wall were <20, mouse was filmed for an additional 5 min. Data were analyzed by formula, where “both” paws was defined as touches where the animal landed both of the forepaws on the cylinder wall at the same time. Intact control mice should score ~50% in this test. No habituation of the animals to the testing cylinder was allowed before the experiment. One animal from aSyn injected and vehicle treated group was excluded from behavioral analyses for scoring too low in three out of five measurement times (<10 touches per record session).

4.4.5 OPENFIELD ACTIVITY TEST

The spontaneous locomotor activity of the AAV-aSyn and AAV-aSyn + AAV-PREP injected mice was measured before the AAV-injections and every 2-3 weeks after the injections for 13 weeks. The animals were placed in transparent cages (30 cm × 30 cm) with the activity monitor measuring infrared beam interruptions (MED Associates, GA, USA). Total distance (locomotor activity) and vertical activity were analyzed. Data was collected in 5 min intervals and activity was recorded for 2 hours.

4.4.6 CONVENTIONAL MICRODIALYSIS

Microdialysis was performed in the end of the PREP inhibitor treatment to AAV-GFP and AAV-aSyn injected mice as described earlier (Käenmäki *et al.* 2010). A microdialysis probe (1-mm cuprophan membrane, o.d. 0.2 mm, 6

kDa cut-off; AT4.9.1. Cu,AgnTho's, Sweden) was inserted into the guide cannula 2 hours prior to the experiment and the probe was perfused with a modified Ringer solution (147 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, and 0.04 mM ascorbic acid) at a flow rate of 2.0 µL/min. Five baseline samples were collected (20 min/40 µL/sample) after the stabilization period. After the collection of baseline samples, the probe was perfused 2 × 20 min with 10 and 30 µM d-amphetamine sulphate with 2 × 20 min recovery time between the concentrations.

The mice were euthanized right after the microdialysis experiment by cervical dislocation followed by decapitation. The brains were removed and rapidly frozen in isopentane on dry ice. The concentrations of DA, its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) as well as gamma-aminobutyric acid (GABA) in dialysates were measured using slightly modified versions of the HPLC methods that have been described earlier (Käenmäki *et al.* 2010, Vihavainen *et al.* 2008).

4.4.7 NO-NET-FLUX MICRODIALYSIS

No-net-flux microdialysis was performed with intact PREPko mice, their wild-type littermates, and AAV-aSyn injected and AAV-aSyn + AAV-PREP coinjected PREPko and wild-type mice. Stabilization of the probe was performed as with the conventional microdialysis but the flow rate for probe perfusion was 0.6 µL/min. During the experiment, four concentrations of DA in Ringer solution (C_{in}; 0, 2, 10, and 20 nM) were perfused through the probe in a random order, and 3 × 30 min samples were collected at each concentration for HPLC analysis. Linear equation was constructed for each animal by plotting the net flux of DA through the probe ($DA_{in} - DA_{out}$) against DA_{in}, where DA_{out} is the dialysate DA concentration acquired during the perfusion and DA_{in} is the DA concentration of the perfusion fluid. Based on this equation, the extracellular DA level (DA_{ext}) and the *in vivo* extraction fraction (E_d, the slope of the linear regression line) was calculated as described by (Parsons & Justice 1992). The DA_{ext} value stands for the perfusion fluid DA concentration at which there is no-net-flux of DA through the probe ($DA_{in} - DA_{out} = 0$). E_d has been shown to describe combined DAT, NET, and uptake$_2$ functions (Chefer *et al.* 2006, Chefer *et al.* 2009, Smith & Justice 1994, Justice 1993).

4.4.8 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

Concentration of DA, its metabolites and 5-HIAA in the dialysate was measured by HPLC with electrochemical detection as earlier described (Käenmäki *et al.* 2010) with slight modifications and the concentration of GABA was measured by HPLC with fluorescence detection as earlier described (Vihavainen *et al.* 2008) with slight modifications.

HPLC system for determination of extracellular concentration of DA, its metabolites and 5-HIAA consisted of a solvent delivery pump (Jasco model PU-2080 Plus, Jasco International Co, Japan), pulse damper (SSI LP-21, Scientific Systems, State College, PA, USA), a refrigerated autosampler (Shimadzu SIL-20AC Autosampler, Shimadzu Co, Japan), an analytical column (Kinetex C-18 5 µm, 4.60 x 50 mm, Phemomenex Inc, USA) thermostated by a column heater (CROCO CIL, Cluzeau Info-Labo, France and LaChrom L-7350, Merck, Germany), an electrochemical detector (ESA Coulochem II detector, ESA Biosciences) and a model 5014B microdialysis cell (ESA Biosciences). The mobile phase consisted of 0.1 M NaH2PO4 buffer (Merck), 8 % (v/v) methanol (Merck), 0.2 M ethylenediaminetetraacetic acid, 100 mg/l octanesulphonic acid, pH 4.0 and the flow rate was 1.0 ml/min. DA was reduced with an amperometric detector (potential -120 mV against an Ag/AgCl reference electrode) after being oxidized with a coulometric detector (+300 mV); DOPAC and HVA were oxidized with the coulometric detector. Injection volume was 20 µL for conventional microdialysis samples and 15 µL for no-net-flux microdialysis samples. The column temperature was kept at + 45 °C. The chromatograms were processed by AZUR chromatography data system software (Cromatek, Essex, UK).

The HPLC system for determination of the extracellular concentration of GABA consisted of a solvent delivery pump (Jasco model PU-1580 HPLC Pump, Jasco International Co, Japan) connected to an online degasser (Jasco 3-Line Degasser, DG-980-50) and a ternary gradient unit (Jasco LG-1580-02), a refrigerated autosampler (Shimadzu NexeraX2 SIL-30AC Autosampler, Shimadzu Corp), an analytical column (Kinetex C-18 5 µm, 4.60 x 50 mm, Phemomenex Inc) protected by a 0.5-mm inlet filter and thermostated by a column heater (CROCO-CIL, Cluzeau Info-Labo, France), and a fluorescence detector (Jasco Intelligent Fluorescence Detector model FP-1520). The wavelengths of the fluorescence detector were set to 330 (excitation) and 450 (emission). The mobile phase consisted of 0.1 M NaH_2PO_4 buffer (Merck), pH 4.9 (adjusted with Na_2HPO_4), 20 % (v/v) acetonitrile (Merck), and the flow-rate was 1.2 ml/min. Automated sample derivatization was carried out using the autosampler at + 8 °C. The autosampler was programmed to add 6 µL of the derivatizing reagent (3 µl of mercaptoethanol ad 1 ml of o-phtaldialdehyde) to 15 µl of a microdialysis sample, to mix two times, and to inject 20 µL onto the column after a reaction time of 1 min. The chromatograms were processed by AZUR chromatography data system software (Cromatek, Essex, UK).

4.5 TISSUE ANALYSES

4.5.1 ANALYSIS OF BRAIN NEUROTRANSMITTERS AND METABOLITES

Striatal tissue samples were punched below the corpus callosum +0.74 mm from the bregma to 2 mm depth by using sample corer (i.d. of 2 mm) with a plunger (Stoelting Co., USA) on a cryostat (Leica CM3050) and the samples from the SN were punched from a brain slice (thickness 1–1.5 mm) –2.8 mm from the bregma by using sample corer (i.d. of 1 mm) with a plunger (Stoelting Co.). Tissue processing and HPLC analysis of the concentrations of DA, its metabolites DOPAC and HVA, and 5-HIAA in the tissue were performed as described earlier by (Airavaara *et al.* 2006).

4.5.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

Tissue concentration of DA, 5-HT and their metabolites were measured as earlier described (Airavaara *et al.* 2006) with slight modifications. The HPLC system consisted of a solvent delivery pump (ESA Model 582, ESA Biosciences), a refrigerated autosampler (Shimadzu SIL-20AC, Shimadzu Corp, Japan), a microdialysis cell (ESA Model 5014B, ESA Biosciences), an analytical column (Kinetex C-18 2.6 µm, 4.6 x 50 mm, Phenomenix Inc, USA) thermostated by a column heater (CROCO-CIL, Cluzeau Info Lab), a pulse damper (SSI LP-21, Scientific Systems, State College, PA, USA) and an electrochemical detector (ESA CoulArray 5600A, ESA Biosciences). The mobile phase consisted of 0.1 M NaH_2PO_4 buffer, 200 mg/l of octane sulfonic acid, methanol (10 %), and 0.2 M EDTA, and the flow-rate was 1.0 µl/min. Injection volume was 100 µl. The column temperature was kept at + 45 °C. Chromatograms were processed and concentrations of monoamines calculated using CoulArray for Windows software (ESA Biosciences, Chelmsford, USA).

The tissue concentrations of GABA and glutamate were analyzed with HPLC equipped with a fluorescence detector. Injection volume was 10 µL and mobile phase consisted of 15 % (v/v) acetonitrile in glutamate measurement, otherwise the method was similar to GABA analysis of microdialysis samples as described above.

4.5.3 WESTERN BLOTTING

Western blotting was performed to measure expression level of TH, the rate limiting enzyme in DA synthesis, total DAT expression and phosphorylated DAT in the STR and SN of PREPko mice, their wild-type littermates, and AAV-PREP or AAV-GFP injected mice. Frozen brain tissue was homogenized with an ultrasound sonicator (GM35-400, Rinco Ultrasonic, Switzerland) in five

volumes of ice-cold modified RIPA buffer (50 mM Tris HCl pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl) containing Halt Phosphatase Inhibitor (Product# 87786, Thermo Fisher Scientific, IL, USA) and Halt Protease Inhibitor cocktail (Product# 78430, Thermo Fisher Scientific). After centrifuging at 16,000g and + 4°C for 15 min, the supernatants were collected. Protein concentration was measured by the BCA method (Thermo Fisher Scientific). For SDS-PAGE, 30 µg of protein was loaded onto a 4–20 % Mini-PROTEAN TGX precast gel (Product # 4561094, Bio-Rad, CA, USA). Gel was transferred to nitrocellulose membrane (Trans-blot Turbo, Product# 1704158, Bio-Rad) by using Trans-blot Turbo Transfer System (BioRad). The membranes were blocked by 5 % skim milk. Western blotting for DAT, TH, and β-actin were done with SNAP i.d. 2.0 protein detection system (Merck Millipore, MA, USA), except phospho (T53)-DAT that was done by incubating the membrane with primary antibody overnight at + 4 °C and thereafter with secondary antibody for 2 h at room temperature. The following primary antibodies and dilutions were used: TH, rabbit anti-TH (#ab153, Merck Millipore, dilution 1:2000 in 0.5 % skim milk in 0.1 % TTBS); β-actin, rabbit anti-β-actin (#4967S, Signaling Technology, MA, USA, dilution 1:1000 in 0.5 % skim milk in 0.1 % TTBS). DAT and pDAT antibodies and dilutions were similar to the cell samples in section 4.3.2 but the antibodies were diluted to 0.5 % skim milk in 0.1 % TTBS. Goat anti-rabbit HRP was used as the secondary antibody for all the primary antibodies (#31460, Thermo Fisher Scientific, dilution 1:2000 in 0.5 % skim milk in 0.1 % TTBS). The images were captured with LI-COR C-digit chemiluminescence scanner (LI-COR, Germany). Three independent Western blotting experiments were performed. OD values of the Western blotting images were analyzed as with cellular Western blotting in the section 4.3.2.

4.5.4 IMMUNOHISTOCHEMISTRY

TH+ neurons in the SN and their TH+ positive fibers in the STR, and oligomeric aSyn were studied by immunohistochemistry staining in the AAV-aSyn or AAV-GFP injected mice that received 4-week minipump treatment with KYP-2047 or vehicle. TH+ neurons, oligomeric aSyn and total aSyn were measured in the STR and SN of aSyn or aSyn+PREP injected wild-type and PREPko mice.

Mice were deeply anesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with PBS followed by 4 % paraformaldehyde (PFA) in PBS. Brains were postfixed for 24–72 h in 4 % PFA at + 4 °C and transferred to a solution of 10% sucrose in PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) overnight at + 4 °C. On the next day, tissue was transferred to 30 % sucrose solution in PBS until brains sank. Brains were frozen on dry ice and were kept at -80°C until sectioning. Frozen brain sections were sectioned as 30 µm free-floating sections on a cryostat (Leica CM3050) and kept in a cryoprotectant solution (30 % ethylene glycol and 30 % glycerol

in 0.5 M phosphate buffer). Thereafter, brains were frozen in isopentane on dry ice and kept at -80°C until further analyses.

TH immunohistochemistry staining was modified from the study by (Mijatovic *et al.* 2007). Shortly, the endogenous peroxidase activity was inactivated with 10% methanol and 3% hydrogen peroxide (H_2O_2) solution in PBS, pH 7.4, for 10 min, and nonspecific binding was blocked with 10% normal goat serum (catalog #S30, Millipore) in PBS containing 0.5% Triton X-100. The sections were incubated overnight at room temperature with rabbit anti-TH primary antibody (1:2000; AB152, Millipore) in 1 % normal goat serum in PBS containing 0.5 % Triton X-100. On the following day, the sections were washed with PBS containing 0.5 % Triton X-100 and then incubated with goat anti-rabbit biotin-conjugated secondary antibodies (1:500; BA1000, Vector Laboratories) in 1% normal serum in PBS containing 0.5 % Triton X-100. The signal was enhanced with the avidin– biotin complex method (Standard Vectastain ABC kit, Vector Laboratories) followed by incubation with 0.05 % 3,3´-diaminobenzidine (DAB) and 0.03 % H_2O_2 solution. Finally, the sections were transferred to glass slides, dehydrated in alcohol series, and mounted with Depex (BDH Chemicals, UK).

Total aSyn immunohistochemistry staining was performed as described in (Myöhänen *et al.* 2012). Shortly, non-specific binding was blocked with 10% normal donkey serum (#S30, Millipore, Temecula, CA, USA). The sections were incubated overnight at room temperature with sheep anti-aSyn primary antibody (1:500; ab6162, AbCam, Cambridge, UK). The sections were then incubated with donkey anti-sheep HRP conjugated secondary antibody for 2 h (1:500; ab6900, AbCam). The antigen–antibody complexes were identified with DAB.

Oligomer-specific aSyn immunohistochemistry staining was performed using the Basic Vector Mouse on Mouse (M.O.M.) Immunodetection Kit (BMK-2202, RRID:AB_2336833, Vector Laboratories) with an adapted protocol for mouse primary antibodies on mouse tissue (Brännström *et al.* 2014). In aSyn oligomer staining, after blocking endogenous peroxidase activity (as above), the sections were incubated for 30 min in M.O.M. Mouse Ig Blocking Reagent to block nonspecific binding and 5 min in M.O.M. diluent, and transferred overnight in mouse anti-human aSyn oligomer-specific primary antibody (1:200 in M.O.M. diluent; AS132718, Agrisera). The sections were then incubated with goat anti-mouse HRP-conjugated secondary antibody (dilution, 1:300 in M.O.M. diluent, catalog #31430, Thermo Fisher Scientific) and visualized with DAB.

4.5.5 MICROSCOPY AND STEREOLOGY

ODs of TH stained ipsilateral and contralateral STR and SN were measured. Digital images were scanned at 40× magnification with a Pannoramic Flash II Scanner (3DHISTECH, Budapest, Hungary), and three coronal sections from each mouse were processed for further analyses with Pannoramic Viewer

(version 1.15.3., 3DHISTECH). Images were converted to grayscale and inverted, and line analysis tools for STR or freehand for SN in ImageJ (NIH) was used to measure the OD of immunoreactivity. To correct the effect of TH background staining, correction values were obtained from the corpus callosum of each section and subtracted from the OD values of the STR. The data are presented as percentages of the intact side.

The number of TH+ cells in SN pars compacta (SNpc) was estimated using the optical fractionator method in combination with the dissector principle and unbiased counting rules (Gundersen *et al.* 1999). The SNpc was analyzed with a Stereo Investigator platform (MicroBrightField, Williston, VT, USA) attached to an Olympus BX51 microscope. From each animal, three sections from the central portion of the SNpc were selected for quantitative analysis. Each reference space was outlined at low magnification (4×), and cells were counted using a high-magnification (60× UPlanApo, 1.4 numerical aperture, oil immersion) objective. The grid size was 100×80 μm, and the counting frames were 60 × 60 μm. Injected and intact SNpc (internal control) was counted, no cell loss was observed in the noninjected side. The coefficient of error, calculated according to the procedure of (Schmitz & Hof 2005), was between 0.05 and 0.10. Results are expressed as the mean cell number per section; all stereological estimations were blinded.

4.6 FAST-SCAN CYCLIC VOLTAMMETRY

PREPko and wild-type littermate male mice (5-6 months of age) were decapitated and 300 μm thick coronal brain slices containing the cortex and the STR were cut on a vibratome (7000 smz-2, Campden Instruments, England) in ice-cold cutting saline containing 125 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 0.3 mM KH_2PO_4, 3.3 mM $MgSO_4$, 0.8 mM NaH_2PO_4, and 10 mM glucose. Slices were allowed to recover for 1–2 hours at + 32 °C in a holding chamber with oxygen-bubbled (95 % O2, 5 % CO2) recording saline containing 125 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 0.3 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.8 mM NaH_2PO_4, and 10 mM glucose. In the recording chamber the slices were continuously perfused with + 35 °C oxygen-bubbled recording saline. Fast-scan cyclic voltammetry recordings were performed with cylindrical 5 μm carbon fiber electrodes positioned at the dorsal STR ~50 μm below the slice surface. Striatal slices were electrically stimulated using a bipolar stainless steel electrode placed at a distance of ~100 μm from the recording electrode. Square pulses of 0.2 sec duration were produced by DS3 stimulator (Digitimer Ltd, UK) that was triggered by a Master-8 pulse generator (A.M.P.I., Israel). Stimulus magnitude was selected by plotting a current–response curve to single pulse stimulations and selecting the minimum value that reliably produced the maximal response. Triangular voltage ramps from −450 mV holding potential to +900 mV over 9 ms (scan rate of 300 mV/ms) were applied to the carbon fiber electrode at 100 ms

intervals. Current was recorded with an Axopatch 200B amplifier (Molecular Devices LLC, CA, USA) filtered with 5 kHz low-pass Bessel filter and digitized at 40 kHz (ITC-18 board, InstruTech, NY, USA). Triangular wave generation and data acquisition were controlled and the recorded transients were characterized by a computer routine in IGOR Pro (WaveMetrics Inc,OR, USA) (Mosharov & Sulzer 2005, Mosharov 2008). Background-subtracted cyclic voltammograms, obtained with 1 µM DA solution (DA-HCl), were used to calibrate the electrodes. The DA terminals were stimulated with single electrical pulses at 2 min intervals or by a burst stimulation of 5 pulses at 20 Hz, to study the release probability of the terminals.

4.7 STATISTICAL ANALYSIS

Statistical analysis was performed using either GraphPad Prism (version 6.02, GraphPad Software, Inc., CA, USA) or SPSS Statistics (Version 22.0.0.1 IBM Corporation, NY, USA) software. One-way ANOVA, two-way ANOVA and repeated measures ANOVA with Tukey´s or Bonferroni´s post-hoc test were used as statistical tests. Data are presented as mean ± standard error of the mean (SEM). The results were considered to be statistically significant at $p < 0.05$.

5 RESULTS

PREP has been shown to participate in regulation of the nigrostriatal DAergic system in aSyn transgenic mice (Savolainen *et al.* 2014) and PREP inhibitors can modulate striatal DA level in the rat brain (Jalkanen *et al.* 2012). Based on these findings we decided to further study the interaction between DA and PREP. We studied the mechanisms of PREP as a regulator of DAT in wild-type HEK-293 cells and stable PREPko cells. Inhibition, absence and overexpression of PREP and restoring PREP function to PREPko cells was studied by Western blotting, a DA uptake assay, and a PKC activity assay (Study I).

PREPko mice were used to study the role of PREP in the nigrostriatal DAergic pathway *in vivo*. Absence, overexpression and inhibition of PREP was studied in PREPko mice and wild-type littermates by no-net-flux microdialysis, fast-scan cyclic voltammetry, HPLC tissue analysis, and Western blotting (Study II).

Additionally, as PREP enhances the aggregation of aSyn – the key player in PD cellular toxicity – and PREP inhibition can decrease this process, we wanted to study the impact of PREP inhibitor treatment on DAergic signaling in the AAV-aSyn mouse model of PD. The effect of the treatment on nigrostriatal DAergic function was studied by conventional microdialysis and tissue HPLC analysis. In addition, behavioral tests and immunohistochemistry were used to evaluate aSyn toxicity and neuroprotective effects of a PREP inhibitor (Study III). Since the PREP inhibitor had a beneficial effect in the aSyn mouse model in Study III, we studied if the toxicity of aSyn and its negative impact on nigrostriatal DAergic neurotransmission is dependent on PREP expression in publication IV.

5.1 PREP AND A-SYNUCLEIN AS REGULATORS OF DOPAMINE TRANSPORTER FUNCTION IN HEK-293 CELLS

PREP inhibitor KYP-2047 has been shown to decrease tissue concentration of DA in the STR of A30P point-mutated transgenic mice and immunoreactive DAT in the STR of aSyn transgenic and wild-type mice (Savolainen *et al.* 2014). Phosphorylation of DAT regulates DAT function as phosphorylated DAT is internalized leading to decreased plasma membrane localization and decreased re-uptake of DA (Vaughan *et al.* 1997, Copeland *et al.* 1996). ERK participates in regulation of DAT phosphorylation (Morón *et al.* 2003) and PREP is involved in ERK phosphorylation and activation (Moreno-Baylach *et al.* 2011, Tenorio-Laranga *et al.* 2013). PKC regulates DAT phosphorylation by activating ERK and also by binding directly to DAT (Vaughan *et al.* 1997, Huff

et al. 1997). Based on these findings we hypothesized that PREP could regulate DAT function via ERK and PKC. We investigated the effect of PREP overexpression, inactive S554A-PREP, lack of PREP and restoring PREP on DAT phosphorylation and function in transfected wild-type HEK-293 cells and stable PREPko HEK-293 cells by Western blotting and by the ^{3}H-DA uptake assay. ERK expression and phosphorylation were also measured to study if changes in DAT function are ERK-mediated. Additionally, we studied if aSyn is able to modify the effect of PREP. The effect of PREP inhibition on PKC activity was measured to investigate if PREP-mediated modulation of DAT function is dependent on PKC.

5.1.1 THE EFFECT OF PREP AND α-SYNUCLEIN ON DOPAMINE TRANSPORTER PHOSPHORYLATION AND FUNCTION, AND ERK PHOSPHORYLATION

Active PREP or inactive S554A-PREP overexpression did not have a statistically significant effect on pDAT/DAT ratio (Figure 5A, $F_{5,23} = 1.030$, $p = 0.430$, one-way ANOVA) or pERK/ERK ratio (Figure 5C, $F_{5,23} = 0.395$, $p = 0.846$, one-way ANOVA) but there was a trend for elevated pDAT/DAT ratio in PREPko cells compared to wild-type cells (Figure 5A, $F_{1,23} = 3.948$, $p = 0.062$, two-way ANOVA). Overexpression of aSyn did not change the pDAT/DAT ratio (Figure 5B, $F_{5,23} = 6.865$, $p = 0.991$, one-way ANOVA, Tukey´s post-hoc test) in wild-type HEK-293 cells or the pERK/ERK ratio (Figure 5D, $F_{5,23} = 0.851$, $p = 0.532$, one-way ANOVA) in wild-type and PREPko HEK-293 cells but aSyn (Figure 5B, ($F_{5,23} = 6.865$, $p = 0.006$, one-way ANOVA, Tukey´s post-hoc test) and combination of aSyn and PREP (Figure 5B, $F_{5,23} = 6.865$, $p = 0.0007$, one-way ANOVA, Tukey´s post-hoc test) lowered the pDAT/DAT ratio in PREPko cells.

Overexpression of PREP or inactive S554A-PREP did not have an effect on DA uptake in wild-type or PREPko HEK-293 cells (Figure 6A). Co-expression of aSyn and DAT (Figure 6B, $F_{5,52} = 3.957$, $p = 0.037$, one-way ANOVA, Tukey´s post-hoc test), and co-expression of PREP, aSyn and DAT (Figure 6, $F_{5,52} = 3.957$, $p = 0.002$, one-way ANOVA, Tukey´s post-hoc test) decreased DA uptake in PREPko cells compared to wild-type HEK-293 cells that were transfected with DAT and mock. There was also a similar trend in mock and DAT transfected PREPko cells (Figure 6B, $F_{5,52} = 3.957$, $p = 0.086$, one-way ANOVA, Tukey´s post-hoc test). Two-way ANOVA revealed a statistically significant difference between wild-type and PREPko cells (Figure 6B, cell line effect; $F_{1,52} = 12.442$, $p = 0.0009$, two-way ANOVA) and between mock and DAT and PREP, aSyn and DAT transfected cells (Figure 6B, treatment effect; $F_{2,52} = 3.442$, $p = 0.037$, two-way ANOVA, Tukey´s post-hoc test).

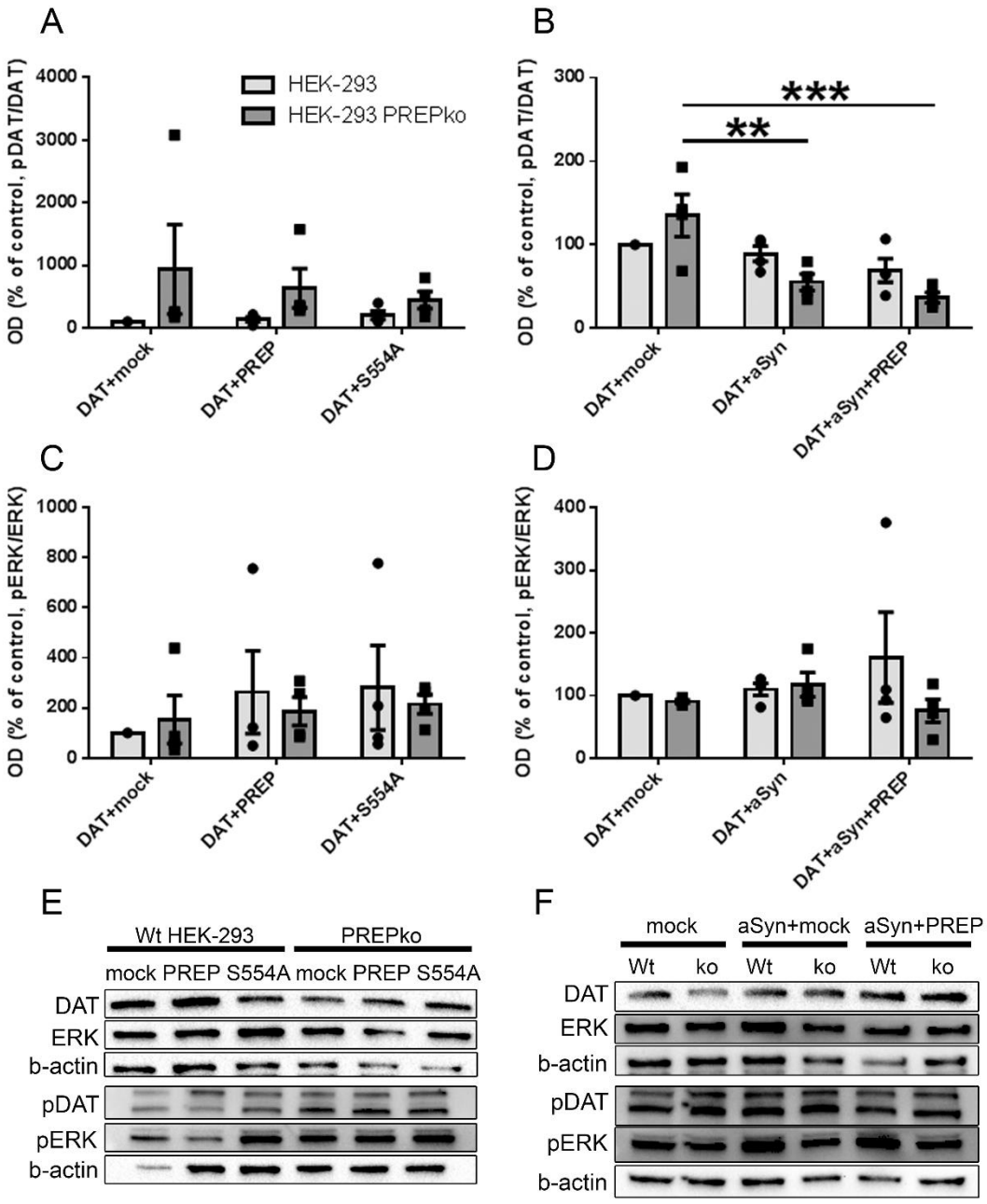

Figure 5 PREP and aSyn regulate phosphorylation of DAT. DAT, pDAT, ERK, and pERK were measured by Western blotting in wild-type HEK-293 and stable PREPko HEK-293 cells transfected with DAT and mock, DAT and PREP or DAT and inactive PREP (S554A-PREP) (A, C and E), or DAT and mock, DAT and aSyn or DAT, aSyn, and PREP (B, D and F). Representative Western blotting figures for A and C (E), and B and D (F). Bars represent mean ± SEM, n = 4, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey´s post-hoc comparison.

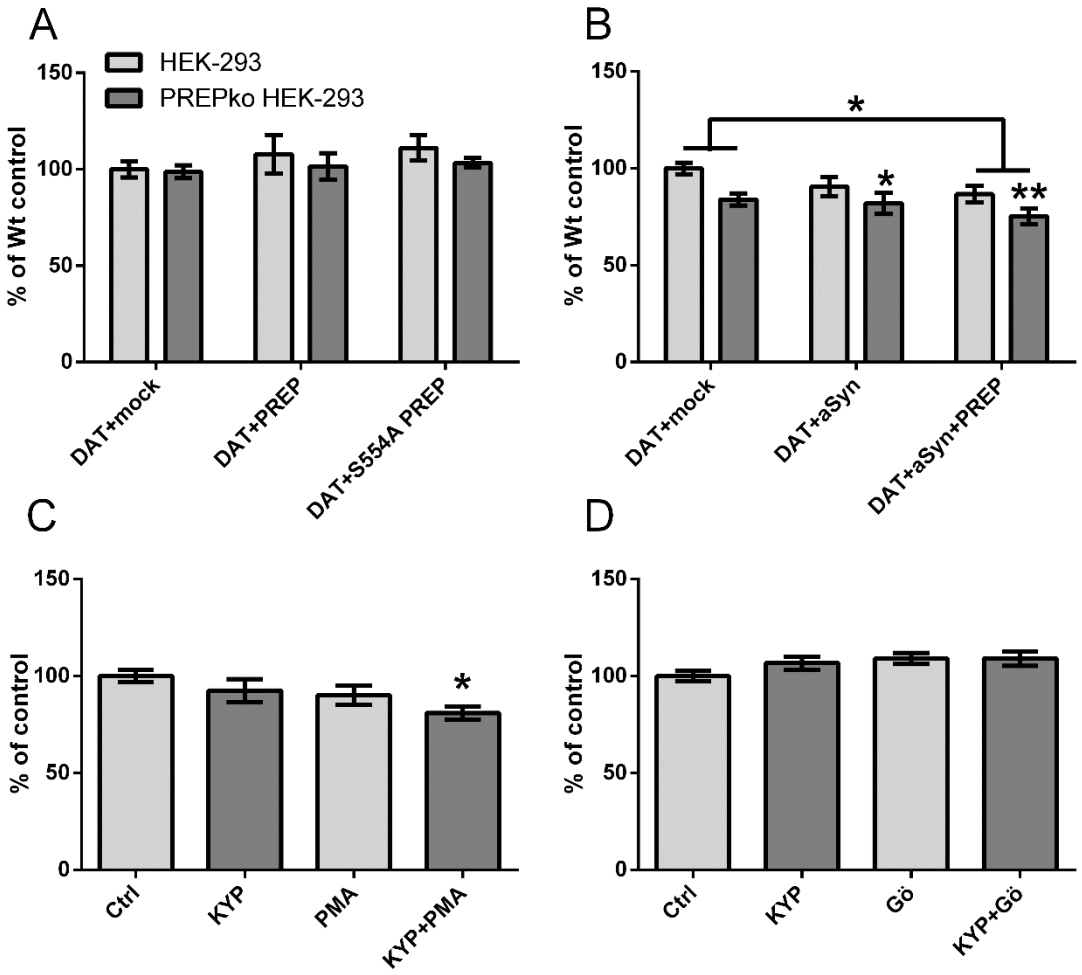

Figure 6 The effect of PREP on DA uptake in wild-type and stable PREPko cells. Uptake of ^{3}H-DA was measured in wild-type and stable PREPko cells transfected with DAT and active or inactive PREP (A), or DAT and aSyn with or without PREP (B) and in DAT transfected wild-type cells that were treated 30 minutes with PREP inhibitor and PKC activator or inhibitor (C, D). Cells were starved in serum free media 2 hours before treatment and then treated with 1 µM KYP, 1 µM PMA or 1 µM KYP + 1 µM PMA for 30 minutes (C). Cells treated with 1 µM KYP, 1 µM Gö-9683 1 µM KYP + 1 µM Gö-6983 for 30 minutes were not starved (D). Data are from 3 independent experiments for cells with 3 replicates. Bars represent mean ± SEM, n = 4, *p < 0.05, **p < 0.01, one-way ANOVA or two-way ANOVA with Tukey´s post-hoc test.

5.1.2 PREP INHIBITOR KYP-2047 POTENTIATES THE EFFECT OF PKC ACTIVATOR PMA

Cells were starved before a PKC activator, PMA, was added to reduce high basal PKC activity which could cover changes in PKC activity. Western blotting and PKC activity assays were performed to measure the impact of PMA and PREP inhibitor (KYP-2047). KYP-2047 treatment did not have an effect on the pDAT/DAT ratio (Figure 7A, $F_{3,15}$ = 6.030, p = 1.000, one-way ANOVA, Tukey´s post-hoc test) or the pERK/ERK ratio (Figure 7C, $F_{3,15}$ = 10.124, p =

1.000, one-way ANOVA, Tukey´s post-hoc test) in starved cells. pDAT/DAT ratio (Figure 7B, $F_{3,15} = 0.886$, $p = 0.736$, one-way ANOVA, Tukey´s post-hoc test) or pERK/ERK ratio (Figure 7D, $F_{3,15} = 4.832$, $p = 0.890$, one-way ANOVA, Tukey´s post-hoc test) were not altered without starvation either. PMA, used as positive control, elevated the pERK/ERK ratio (Figure 7C, $F_{3,15} = 10.124$, $p = 0.018$, one-way ANOVA, Tukey´s post-hoc test) but the decrease in the pDAT/DAT ratio was not statistically significant (Figure 7A, $F_{3,15} = 6.030$, $p = 0.103$, one-way ANOVA, Tukey´s post-hoc test). Combination of KYP-2047 and PMA elevated the pDAT/DAT ratio (Figure 7A, $F_{3,15} = 6.030$, $p = 0.023$, one-way ANOVA, Tukey´s post-hoc test) and the pERK/ERK ratio (Figure 7C, $F_{3,15} = 10.124$, $p = 0.006$ one-way ANOVA, Tukey´s post-hoc test) statistically significantly but the combination did not have a statistically significant difference to PMA alone.

PKC inhibitor Gö-6983 did not have an effect on phosphorylation of DAT in non-starved cells (Figure 7B) but combination of Gö6983 and KYP-2047 decreased phosphorylation of ERK (Figure 7D, $F_{3,15} = 4.832$, $p = 0.029$ one-way ANOVA, Tukey´s post-hoc test). There was also a similar trend in the pERK/ERK ratio with Gö-6983 alone (Figure 7D, $F_{3,15} = 4.832$, $p = 0.087$, one-way ANOVA, Tukey´s post-hoc test).

The effect of PREP inhibition on PKC activity was studied by PKC activity assay since PKC-mediated phosphorylation of DAT is one of the main regulators of DAT function and internalization, and PKC phosphorylates ERK. PMA was used as a positive control and Gö-6983 as a negative control. As predicted, there was a trend for increased PKC activity induced by PMA (Figure 7F, $F_{5,15} = 10.63$, $p = 0.074$, one-way ANOVA, Tukey´s post-hoc test) but the negative control Gö-6983 did not have much effect on PKC activity. However, Gö-6983 was able to abolish the effect of PMA, verifying that PMA-induced signal was indeed PKC activity. KYP-2047 alone did not have an effect on PKC activity but it potentiated the effect of PMA since combination of KYP-2047 and PMA induced a statistically significant increase in PKC activity (Figure 7F, $F_{5,15} = 10.63$, $p = 0.018$, one-way ANOVA, Tukey´s post-hoc test).

Similar potentiation of the effect of PMA was also observed in ^{3}H-DA uptake assay. KYP-2047 alone did not have a statistically significant effect on uptake in starved wild-type HEK-293 cells (Figure 6C, $F_{3,32} = 3.052$, $p = 0.643$, one-way ANOVA, Tukey´s post-hoc test) or without starvation (Figure 6D, $F_{3,31} = 0.517$, $p = 0.455$, one-way ANOVA, Tukey´s post-hoc test). Combination of PMA and KYP-2047 reduced DA uptake (Figure 6C, $F_{3,32} = 3.052$, $p = 0.026$, one-way ANOVA) while PMA alone did not have a statistically significant effect (Figure 6C, $F_{3,32} = 3.052$, $p = 0.413$, one-way ANOVA). PKC inhibitor Gö-6983 did not have a statistically significant effect on DA uptake alone (Figure 6D, $F_{3,31} = 0.517$, $p = 0.180$, one-way ANOVA, Tukey´s post-hoc test) or combined with KYP-2047 (Figure 6D, $F_{3,31} = 0.517$, $p = 0.183$, one-way ANOVA, Tukey´s post-hoc test).

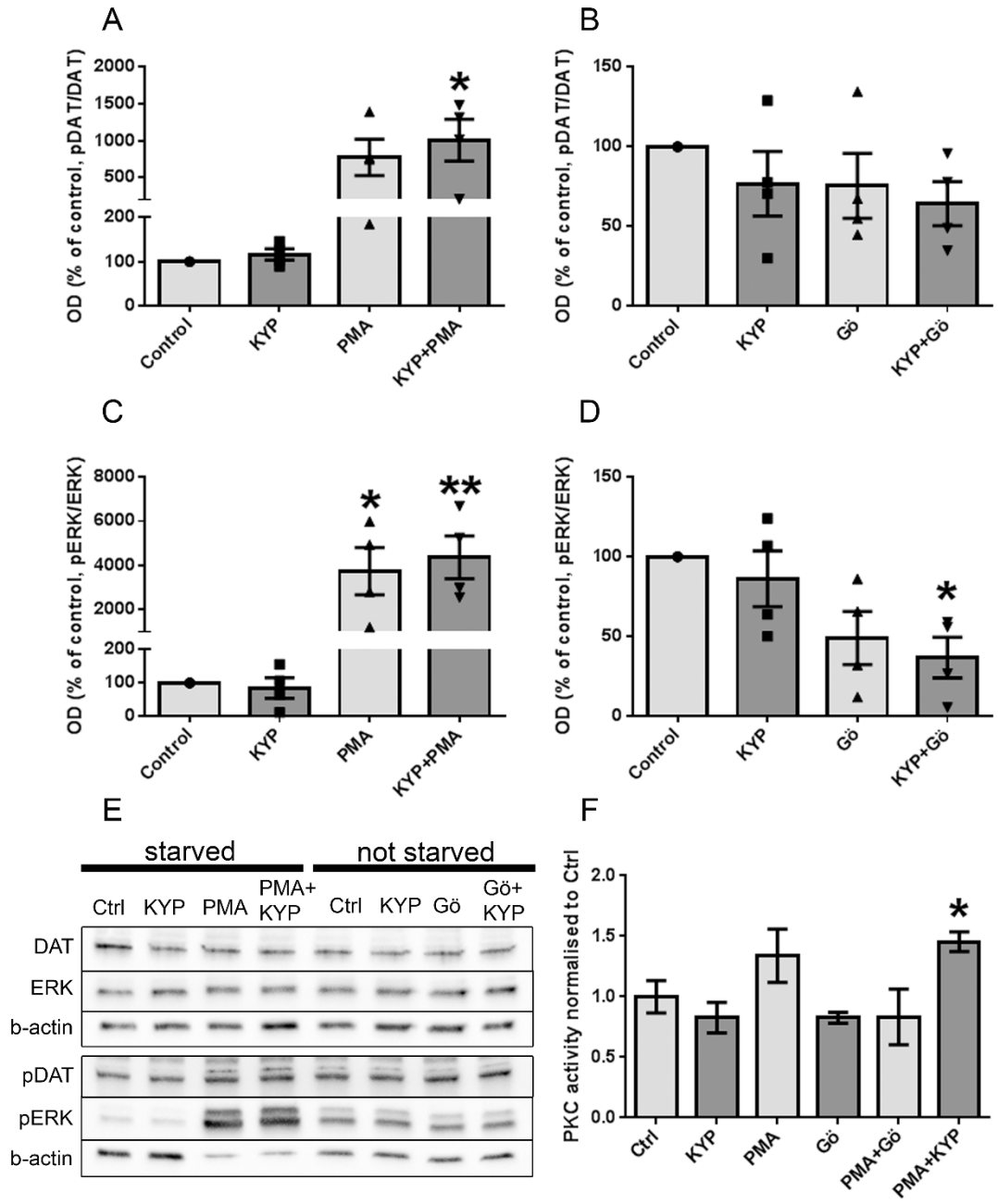

Figure 7 Wild-type HEK-293 cells were starved for 2 hours in serum free media and then treated with 1 µM KYP-2047, 1 µM PMA or 1 µM KYP-2047 + 1 µM PMA for 30 minutes before cell lysis (A, C). Wild-type HEK-293 cells were treated with 1 µM KYP-2047, 1 µM Gö-6983 or 1 µM KYP-2047 + 1 µM Gö-6983 for 30 minutes without starvation (B, D). Representative Western blots for A-D (E). pDAT/DAT ratio and pERK/ERK ratio were measured from Western blots that were normalized to b-actin. pDAT/DAT and pERK/ERK ratio of control cells were set as 100 %. n = 4. Bars represent mean ± SEM, *p < 0.05, **p < 0.01, one-way ANOVA with Tukey´s post-hoc comparison.

5.2 PREP REGULATES DOPAMINE TRANSPORTER PHOSPHORYLATION AND FUNCTION IN THE NIGROSTRIATAL PATHWAY IN MICE

The effect of absence or overexpression of PREP on the DAergic function in the nigrostriatal pathway of mouse was studied in PREPko mice and their wild-type littermates, and in wild-type mice that received supranigral microinjection of AAV-GFP or AAV-PREP. Extracellular DA level was measured by striatal no-net-flux microdialysis, and DA release and uptake were studied in acute striatal slices by fast-scan cyclic voltammetry in PREPko mice and their wild-type littermates. DAT expression and phosphorylation and TH expression in the STR and SN were studied by WB in PREPko animals, wild-type littermates, and AAV-injected mice.

Extracellular DA concentration was elevated in the STR of PREPko mice compared to wild-type littermates in the no-net-flux microdialysis study (Figure 8A, $p = 0.03$, Student´s t-test). Fast-scan cyclic voltammetry revealed that DA release was similar in the STR of PREPko mice and wild-type littermates (Figure 8C, $p = 0.356$, Student´s t-test), but the half widths (Figure 8D, $p = 0.002$, Student´s t-test) and tau values (Figure 8E, $p = 0.028$, Student´s t-test) of the DA release peak were increased in PREPko animals, and there was a similar trend in the fall time (Figure 8F, Student´s t-test) indicating delayed DA re-uptake and impaired DAT function in the PREPko STR.

Lack of PREP did not have an effect on total DAT expression in the STR (Figure 9A) or SN (Figure 9B), but phosphorylated DAT was elevated in the STR of PREPko mice (Figure 9C, $p = 0.034$, Student´s t-test). Changed phosphorylation of DAT was not observed in the SN (Figure 9D). TH expression was similar in the STR of PREPko mice and wild-type littermates (Figure 9E), but elevated TH expression was observed in the SN of PREPko mice (Figure 9F, $p = 0.004$, Student´s t-test).

Overexpression of PREP in the nigrostriatal tract elevated DAT expression in the STR (Figure 10A, $p = 0.027$, Student´s t-test) and in the SN (Figure 10B, $p = 0.033$, Student´s t-test) measured by Western blotting. pDAT was decreased in the STR of PREPko mice (Figure 10C, $p = 0.028$, Student´s t-test), but it was not altered in the SN (Figure 10D). AAV-PREP injection did not have an effect on the striatal (Figure 10E) or nigral TH expression level (Figure 10F).

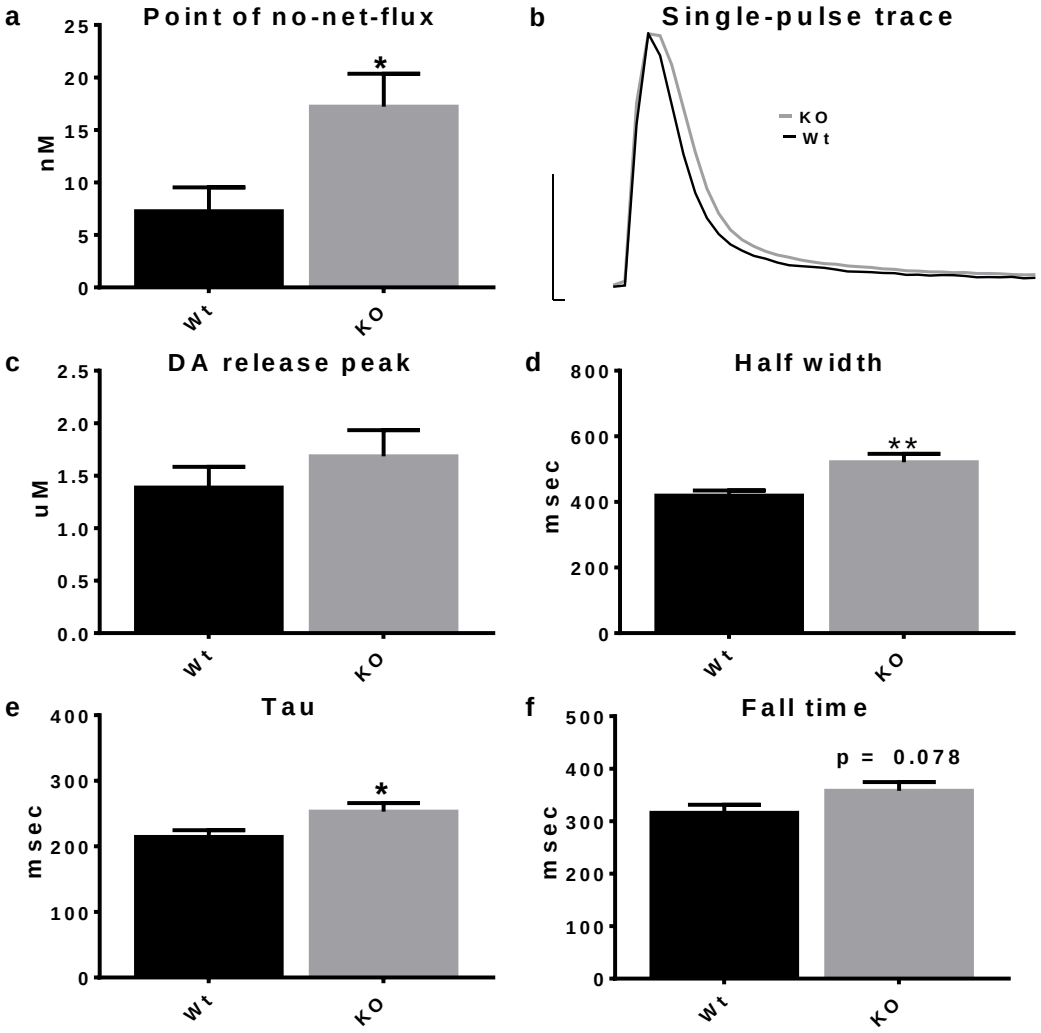

Figure 8 PREPko mice (KO) had higher extracellular DA concentration in the STR than wild-type mice in the no-net-flux microdialysis study (A). Fast-scan cyclic voltammetry revealed delayed re-uptake of DA in PREPko mice compared to wild-type littermates (B). The height of DA release peak was similar in wild-type and PREPko mice (C) but half width (D) and Tau values (E) were elevated in PREPko mice and there was a similar trend in the fall time (F). A : n = 5-6, B-F n = 13-14. Bars represent mean ± SEM, *p < 0.05, **p < 0.01, Student´s t-test.

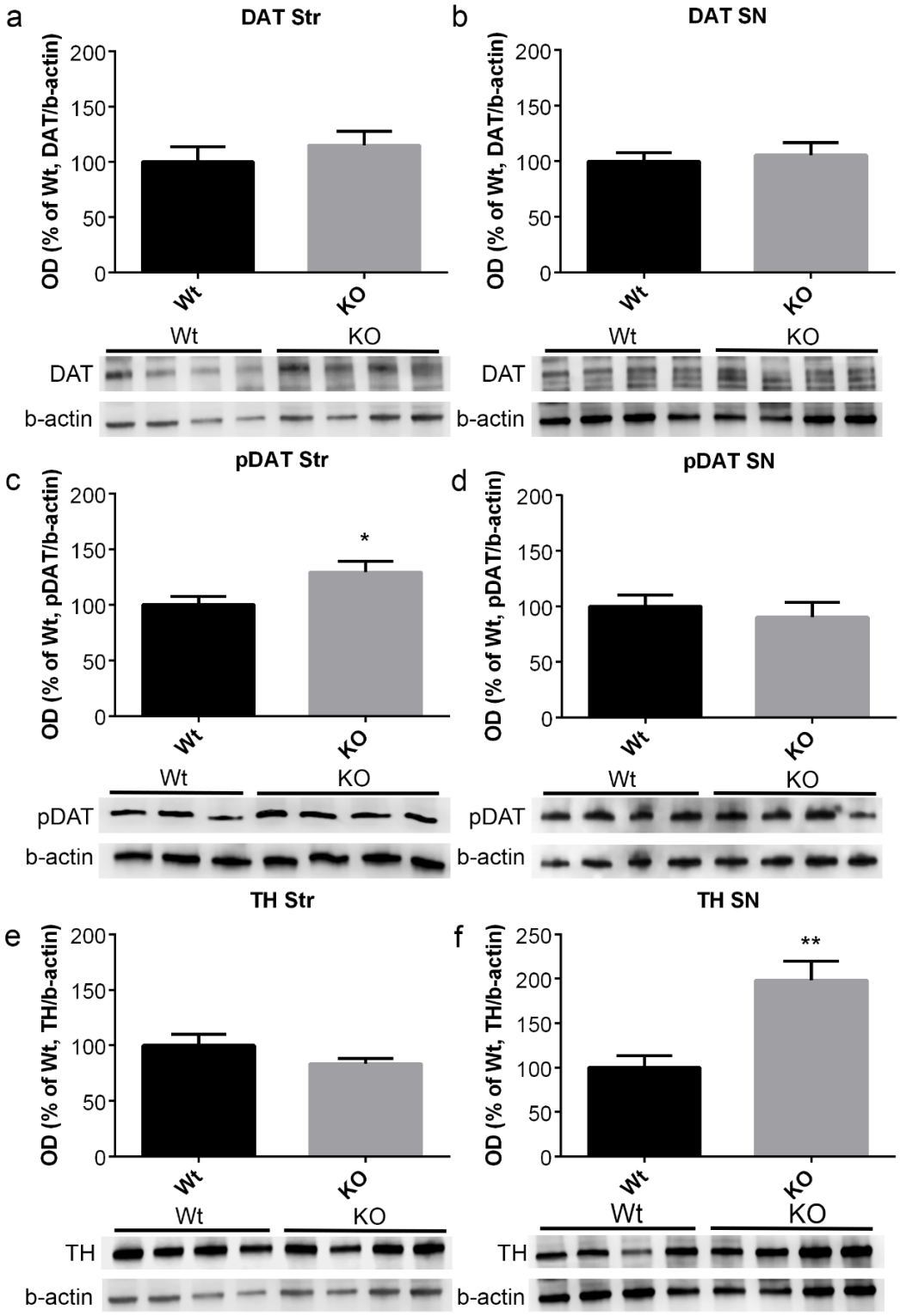

Figure 9 DAT (A-B), pDAT (C-D), and TH (E-F) were measured by Western blotting in the STR and SN tissue of PREPko and wild-type mice. DAT was unchanged in the STR (A) and in the SN (B). pDAT was elevated in the STR (C) but not in the SN (D) in PREPko mice. TH was elevated in the SN (F) but not in the STR (E) of PREPko mice. n = 3-4. Bars represent mean ± SEM, *$p < 0.05$, **$p < 0.01$, Student´s t-test.

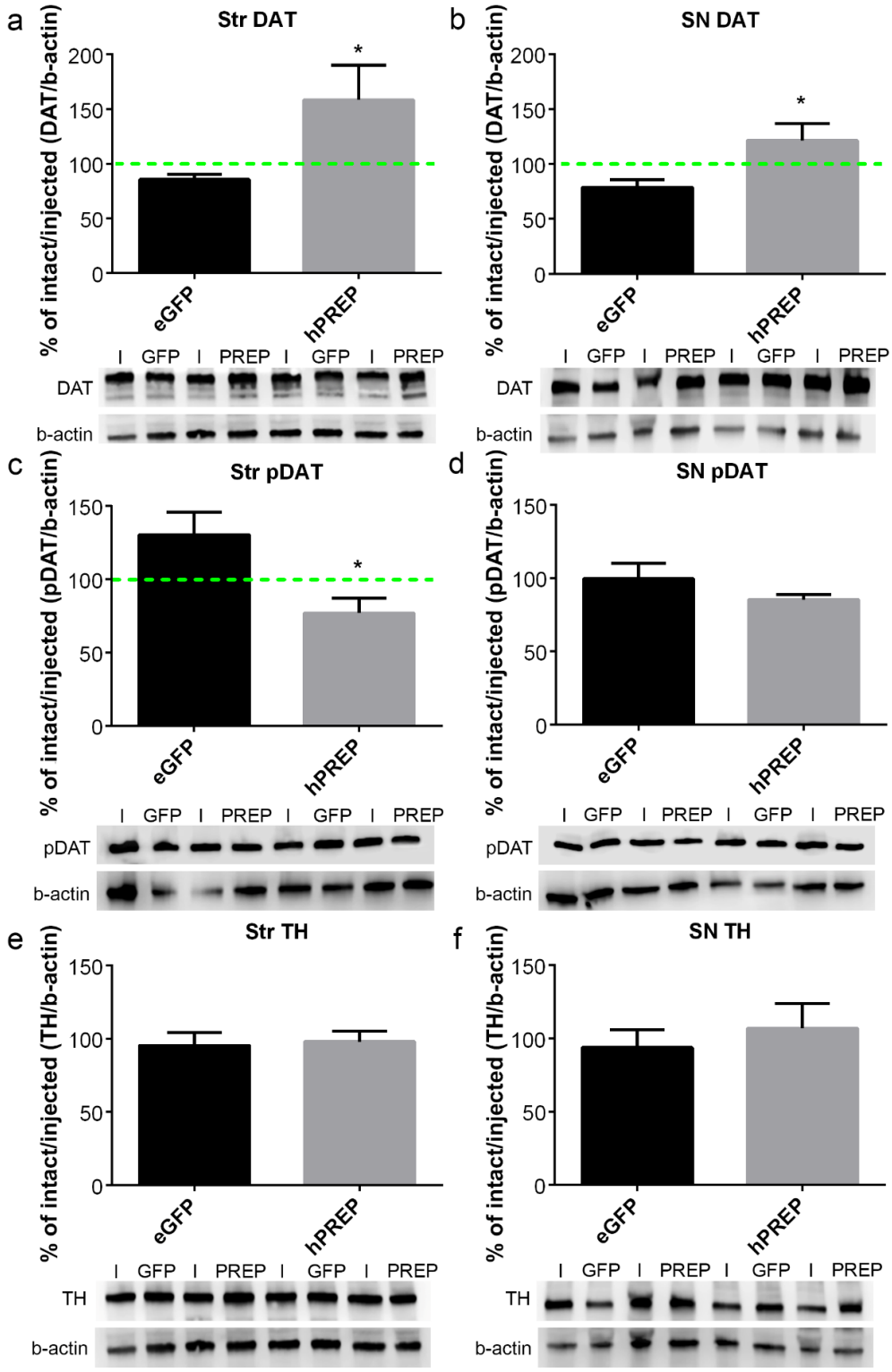

Figure 10 DAT (A-B), pDAT (C-D), and TH (E-F) were measured by Western blotting in the STR and SN tissue 5 weeks after a supranigral injection of AAV-eGFP or AAV-hPREP. Overexpression of PREP increased DAT in the STR (A) and in the SN (B). pDAT was increased in the STR (C) but not in the SN (D). TH was not changed (E-F). n = 3-4. Bars represent mean ± SEM, *p < 0.05, Student´s t-test.

5.3 PREP INHIBITOR RESTORES BEHAVIOR AND DOPAMINERGIC FUNCTION IN A-SYNUCLEIN OVEREXPRESSION MODEL OF PARKINSON´S DISEASE

The effect of PREP inhibitor treatment on behavior and the nigrostriatal DAergic system was studied in the AAV-aSyn mouse model of PD. AAV-aSyn or AAV-GFP were injected above the SN to cause progressive aSyn aggregation and DAergic neurodegeneration to SN and STR to model PD. 4-week KYP-2047 (10 mg/kg per day) or vehicle minipump treatment was started 4 weeks post-injection and the behavior of mice was evaluated every 2 weeks by cylinder test. The effect of aSyn overexpression and KYP-2047 treatment on the nigrostriatal DAergic system was evaluated by TH immunohistochemistry staining to observe cell loss in the STR and SN, by striatal microdialysis to measure extracellular DA and its metabolites, and by tissue HPLC analysis to measure striatal and nigral tissue concentrations of DA and its metabolites.

Unilateral overexpression of aSyn reduced use of the forepaw contralateral to the injected hemisphere at 2 weeks and 4 weeks post-injection compared to AAV-GFP injected control mice (Figure 11, $F_{1,31} = 16.873$. $p = 0.0003$, repeated measures two-way ANOVA). KYP-2047 minipump treatment restored the use of contralateral forepaw but impaired behavior retained in the aSyn-vehicle group (main effect for the treatment, $F_{1,29} = 5.575$, $p = 0.025$, combined treatment by virus F ratio, $F_{1,29} = 6.802$, $p = 0.014$, repeated-measures two-way ANOVA; difference between GFP groups and aSyn-vehicle group, $p < 0.05$, Tukey´s post-hoc test).

aSyn overexpression did not have a statistically significant effect on the cell count of TH+ cells in the SN (Figure 12A, B, $F_{3, 28} = 2.784$, $p = 0.059$, one-way ANOVA), but TH+ OD was reduced in the STR (Figure 12A, C, $F_{3, 29} = 4.229$, $p = 0.014$, aSyn-vehicle vs GFP-vehicle, one-way ANOVA) and in the SN (Figure 12A, D, $F_{3,30} = 7.366$, $p = 0.0008$, one-way ANOVA; $p < 0.01$, aSyn-vehicle vs GFP-vehicle and GFP-KYP, Tukey´s post-hoc test) in the AAV-aSyn injected vehicle treated mice compared to the AAV-GFP injected control mice. Intriguingly, similar reduction was not observed in the AAV-aSyn injected mice that received KYP-2047 treatment (Figure 12A, C, D). Nevertheless, immunohistochemistry showed that KYP-2047 treatment significantly reduced oligomeric aSyn particles in the SN (Figure 12E, $p < 0.0001$, Student´s t-test).

Overexpression of aSyn decreased striatal extracellular DA metabolites DOPAC and HVA statistically significantly (Figure 13B, $F_{1,35} = 4.164$, $p = 0.049$, two-way ANOVA) and there was a similar trend in DA (Figure 13A, $F_{1,35} = 3.758$, $p = 0.061$, two-way ANOVA) in the microdialysis study, but KYP-2047 treatment did not have a significant effect on DA (Figure 13A, aSyn-vehicle vs. aSyn-KYP, $p = 0.129$, Student´s t-test) or its metabolites (Figure 13B, aSyn-vehicle vs. aSyn-KYP, $p = 0.435$, Student´s t-test) in AAV-aSyn injected mice.

Tissue concentration of DA was reduced by aSyn overexpression in the STR (Figure 13C, $F_{1,41} = 6.502$, $p = 0.015$, two-way ANOVA) and there was a similar trend for metabolites of DA (Figure 13D, $F_{1,41} = 3.611$, $p = 0.064$, two-way ANOVA). DA concentration was not changed in SN (Figure 13E), but the metabolites of DA were decreased (Figure 13F, $F_{1,41} = 6.502$, $p = 0.015$, two-way ANOVA).

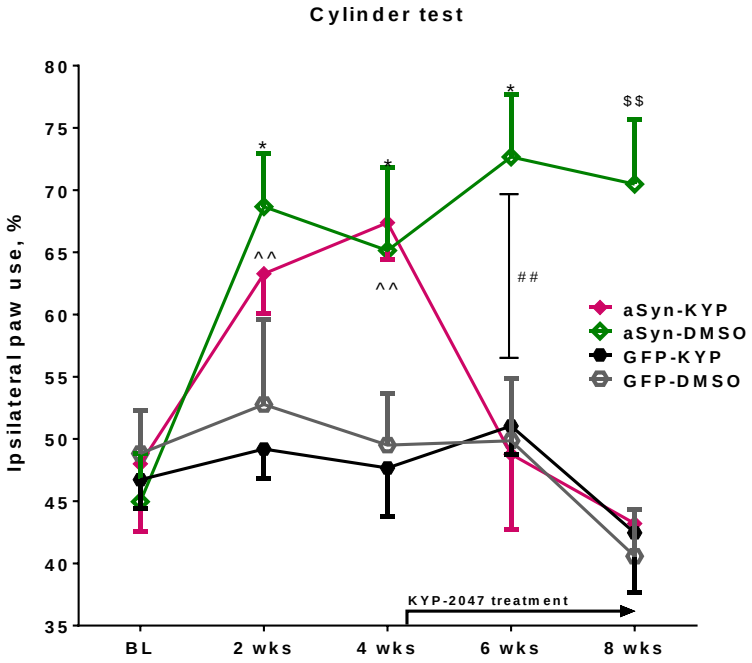

Figure 11 KYP-2047 treatment restores motor behavior in cylinder test in aSyn overexpressing mice. AAV-eGFP or AAV-aSyn were injected above SN and 4-week minipump treatment with vehicle (VEH) or KYP-2047 (KYP) was started 4 weeks post-injection. Motor behavior was evaluated by cylinder test every two weeks. aSyn overexpression caused increased use of ipsilateral paw two weeks after the AAV-injections and KYP-treatment restored use of ipsilateral paw on the baseline level two weeks after the minipump implantation. Until 6 weeks: n = 7-9, at 8 weeks: n = 4-7. Bars represent mean ± SEM. *$p < 0.05$, aSyn-vehicle vs. GFP-vehicle; ^^$p < 0.01$, aSyn-KYP vs. GFP-KYP; ##$p < 0.01$, aSyn-vehicle vs. aSyn-KYP; §§$p < 0.01$, aSyn-vehicle vs. aSyn-KYP, GFP-vehicle, and GFP-KYP, two-way ANOVA with Tukey's post hoc comparison.

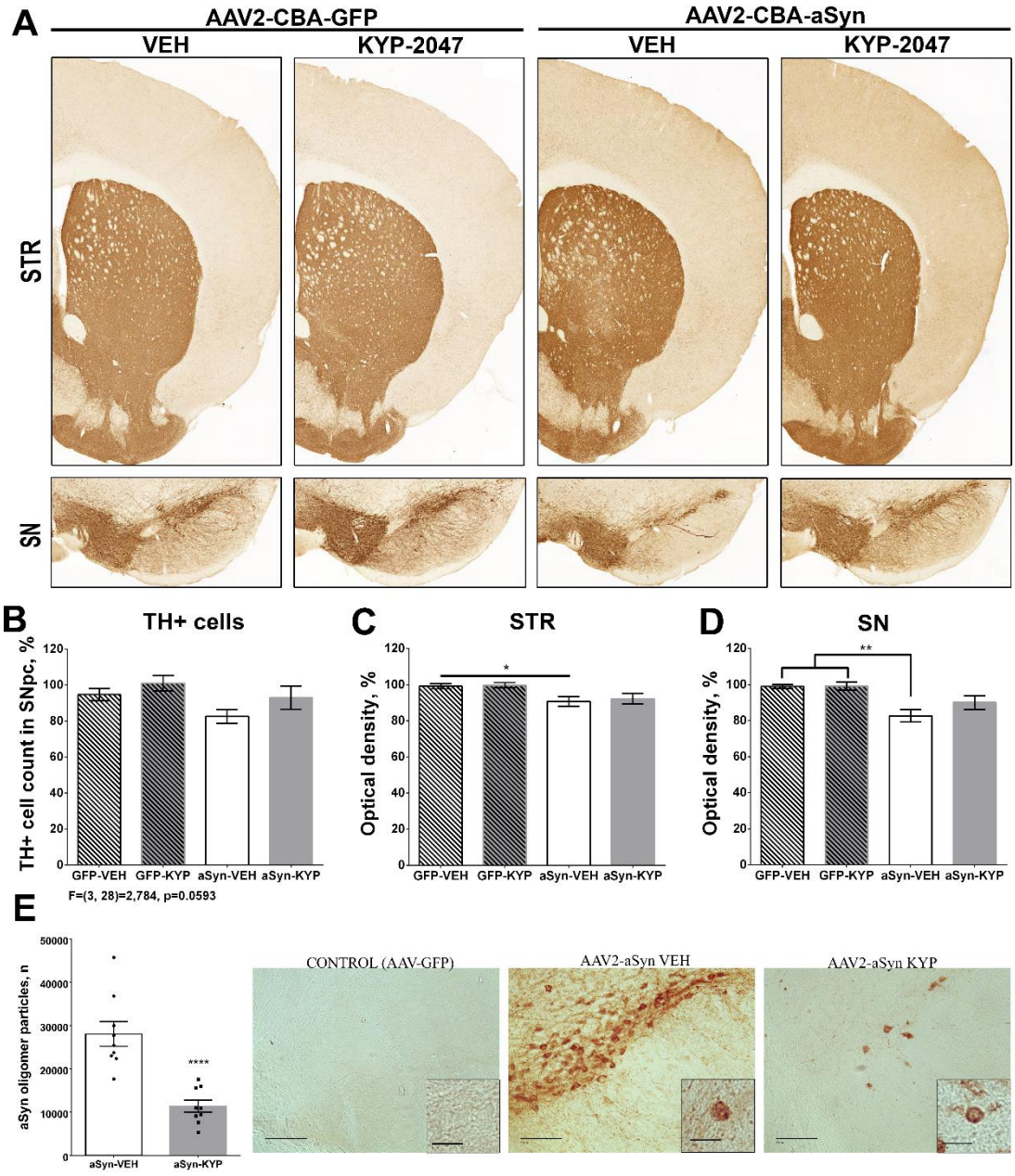

Figure 12 aSyn overexpression decreased TH+ cells and TH immunoreactivity in the STR and in the SN. Mice received supranigral injection of AAV-GFP and AAV-aSyn and 4 weeks post-injection intraventricular 4-week treatment with vehicle (VEH) or KYP-2047. Representative images of TH+ immunoreactivity in the STR and in the SN (A). aSyn did not have a statistically significant effect on the TH+ cell stereology (B) but TH+ OD in the STR (C) and SN (D) was decreased in the vehicle-treated AAV-aSyn injected mice compared to AAV-GFP injected control groups. Similar decrease was not observed in KYP-2047 treated AAV-aSyn injected mice (C, D). 4-week KYP-2047 treatment reduced immunoreactive aSyn oligomer particles in the SN 8 weeks after AAV-aSyn injection compared to AAV-GFP-injected animals (E). n = 7-9. Bars represent mean ± SEM, *p < 0.05, **p < 0.01, ****p < 0.0001, one-way ANOVA with Tukey´s post-hoc comparison (B-D), unpaired Student´s t-test (E). Scale bars: 100 µm; inserts 15 µm.

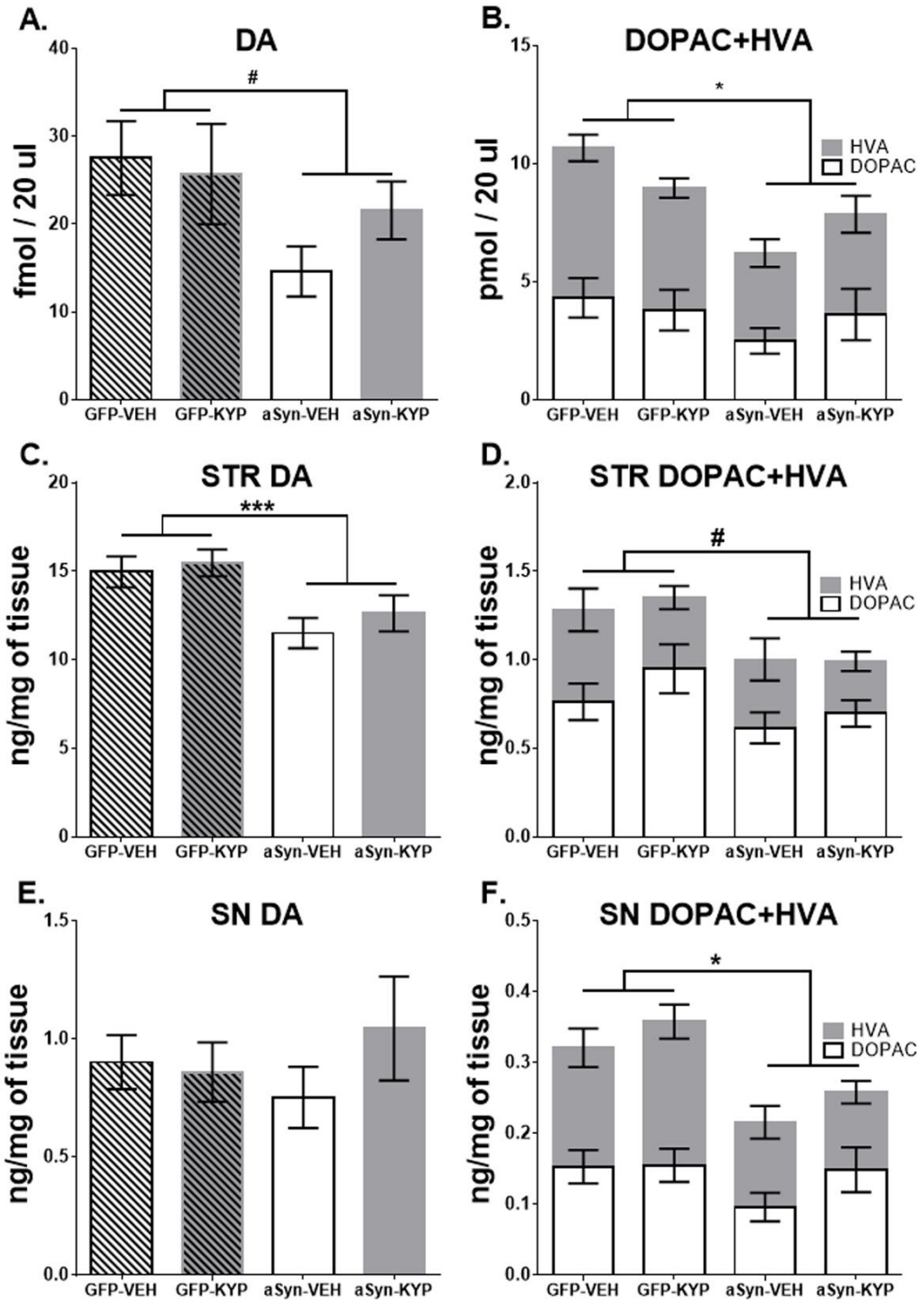

Figure 13 Overexpression of aSyn decreased extracellular and tissue concentration of DA in the STR and its metabolites DOPAC and HVA in the STR and SN in wild-type mice. Mice received supranigral injection of AAV-GFP or AAV-aSyn, and vehicle (VEH) or KYP-2047 minipump treatment was started 4 weeks post-injection. Extracellular level of DA (A) and its metabolites DOPAC and HVA (B) were measured by striatal microdialysis, and striatal (C, D) and nigral (E, F) tissue concentrations by HPLC analysis after 4-week treatment. Overexpression of aSyn lowered DOPAC and HVA statistically significantly (B) and there was a similar trend for DA (A). aSyn decreased DA in the striatal tissue (C) and metabolites in the nigral tissue (F) and there was a similar trend for metabolites in the STR (D) but nigral DA was not affected (E). KYP-2047 treatment did not have a significant effect on DA or its metabolites. n = 8-11. Bars represent mean ± SEM. *$p < 0.05$; ***$p < 0.001$; #$p = 0.06$, two-way ANOVA.

5.4 REMOVAL OF PREP REDUCES α-SYNUCLEIN TOXICITY IN THE MOUSE BRAIN

To characterize the role of PREP on aSyn aggregation and toxicity, and the impact of this on the DAergic system, we restored PREP in the presence and absence of aSyn in the PREPko mouse nigrostriatal pathway, and similarly used overexpression in the wild-type mouse. The nigrostriatal DAergic system was studied by striatal no-net-flux microdialysis and tissue HPLC analysis 14-15 weeks after supranigral injection of AAV-aSyn or co-injection of AAV-aSyn and AAV-PREP to PREPko mice or wild-type littermates. The behavioral effects were investigated by locomotor activity test and cylinder test before the viral vector injections, and starting 2 weeks after the injections animals were tested every 3 weeks until 13 weeks. Distribution of aSyn, and loss of TH+ fibers in the STR and TH+ cells in the SN were evaluated by immunohistochemistry staining of total aSyn, oligomeric aSyn, and TH.

Locomotor activity in PREPko mice was restored to the levels of wild-type animals after the coinjection of PREP and aSyn (Figure 14A).There was a statistically significant interaction between the aSyn and aSyn+PREP injected animals and time on traveled distance in the PREPko mice (Figure 14A, $F_{5, 75} = 4.174$, $p = 0.002$, two-way ANOVA). The traveled distance was decreased in the PREPko mice that received coinjection of aSyn+PREP at the 5-week timepoint (Figure 14A, $F_{1, 15} = 5.612$, $p = 0.032$, univariate analysis) and the difference extended until the last timepoint at 13 weeks (Figure 14A, $F_{1,15} = 7.642$, $p = 0.014$). Similar changes were not observed in the wild-type mice (Figure 14A, $F_{5,70} = 1.002$, $p = 0.395$, two-way ANOVA).

The interaction between the viral vector and ipsilateral paw use was not significant in PREPko mice (Figure 14B, $F_{5,145} = 0.639$, $p = 0.622$, two-way ANOVA) or in wild-type littermates (Figure 14B. $F_{5,150} = 1.696$, $p = 0.139$, two-way ANOVA). However, the main effect over time showed a statistically significant difference in ipsilateral paw use in wild-type mice (Figure 14C, $F_{5,150} = 5.453$, $p = 0.001$, two-way ANOVA with Bonferroni´s adjustment) indicating aSyn-induced toxicity. Paw preference was changed statistically significantly in wild-type mice 2 weeks after the injections compared to baseline level ($p < 0.0005$) and the difference remained until the end of the experiment 13 weeks post-injections (Figure 14B, $p = 0.007$).

Immunohistochemistry staining showed that oligomeric aSyn was not changed statistically significantly in any of the groups (Figure 15B) contrary to observations of PREP inhibitor treatment in Study III. However, TH+ cells in the SN were decreased more in aSyn+PREP co-injected wild-type and PREPko mice than in aSyn injected wild-type and PREPko mice (Figure 15A, $F_{1,23} = 7.965$, $p = 0.0097$, two-way ANOVA). Nevertheless, TH OD analysis did not show loss of TH+ fibers in the STR or in the SNpc (Figure 16D, E, G) but the SNpr two-way ANOVA revealed a statistically significant effect for viral vector injections (Figure 16F, G; $F_{1,25} = 4.838$, $p = 0.037$, two-way ANOVA), and for phenotype (Figure 16C, G; $F_{1,25} = 4.410$, $p = 0.046$, two-way ANOVA).

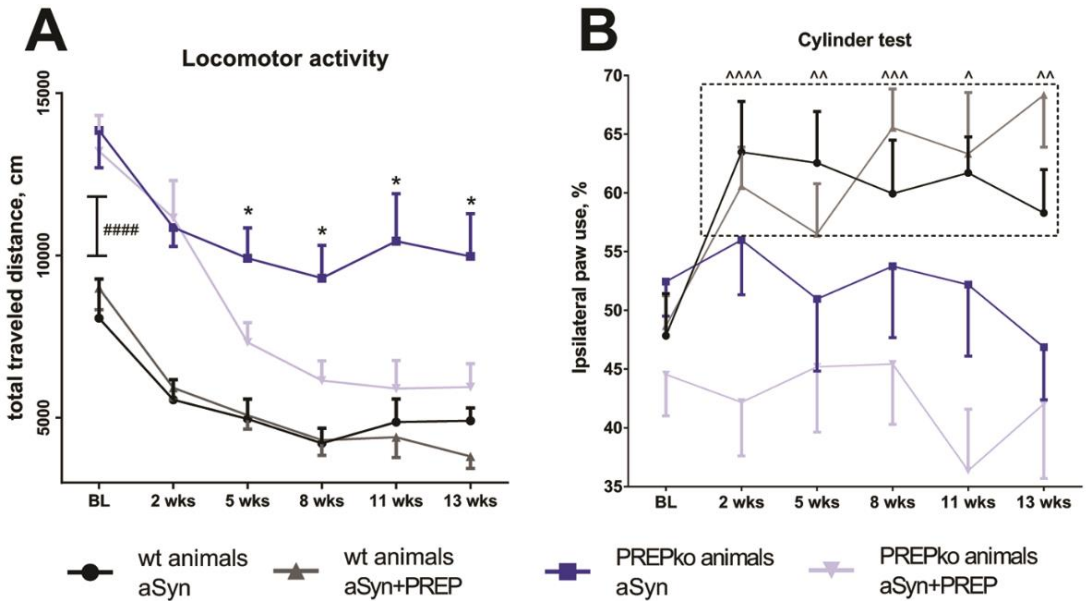

Figure 14 PREPko mice were resistant to the aSyn-induced changes in the behavioral tests. Total traveled distance was significantly reduced in PREPko mice that received aSyn+PREP co-injection compared to PREPko mice that received only aSyn starting 5 weeks after the injections and the difference extended until the end of the experiments. n = 7-10 (A). Use of ipsilateral paw was increased only in the wild-type (wt) mice that received aSyn or aSyn+PREP injection but this difference was not seen in the PREPko mice. n = 15-17 (B). Bars represent mean ± SEM. *p < 0.05, ####p < 0.0005, wild-type vs. PREPko; ^p < 0.05, ^^p < 0.01, ^^^p < 0.001, ^^^^p < 0.0005, wild-type mice baseline (BL) vs. post-injection measurements, two-way ANOVA with univariate analyses, Student´s t-test for BL locomotor activity.

The effect of aSyn and PREP overexpression on extracellular DA was studied by no-net-flux microdialysis and on tissue concentration of striatal DA, its metabolites and GABA by HPLC tissue analysis. The level of extracellular DA was similar in all groups in the no-net-flux microdialysis experiment (Figure 15C). Striatal DA was significantly decreased compared to the intact side of the brain in all other groups (Figure 15D, $F_{7,56} = 9.403$, wt-aSyn p = 0.002, wt-aSyn + PREP p = 0.008, PREPko-aSyn p = 0.007, one-way ANOVA with Tukey's post hoc comparison) except in PREPko mice that received aSyn + PREP injection (Figure 15D, $F_{7,56} = 9.403$, p = 0.218), but DOPAC was increased significantly only in this group (Figure 15E, $F_{7,56} = 3.296$, p = 0.009). AAV-injections did not have an effect on striatal extracellular HVA (Figure 15F) or GABA (Figure 15G).

The main findings of the role of PREP in the nigrostriatal DAergic function are collected together in Table 2.

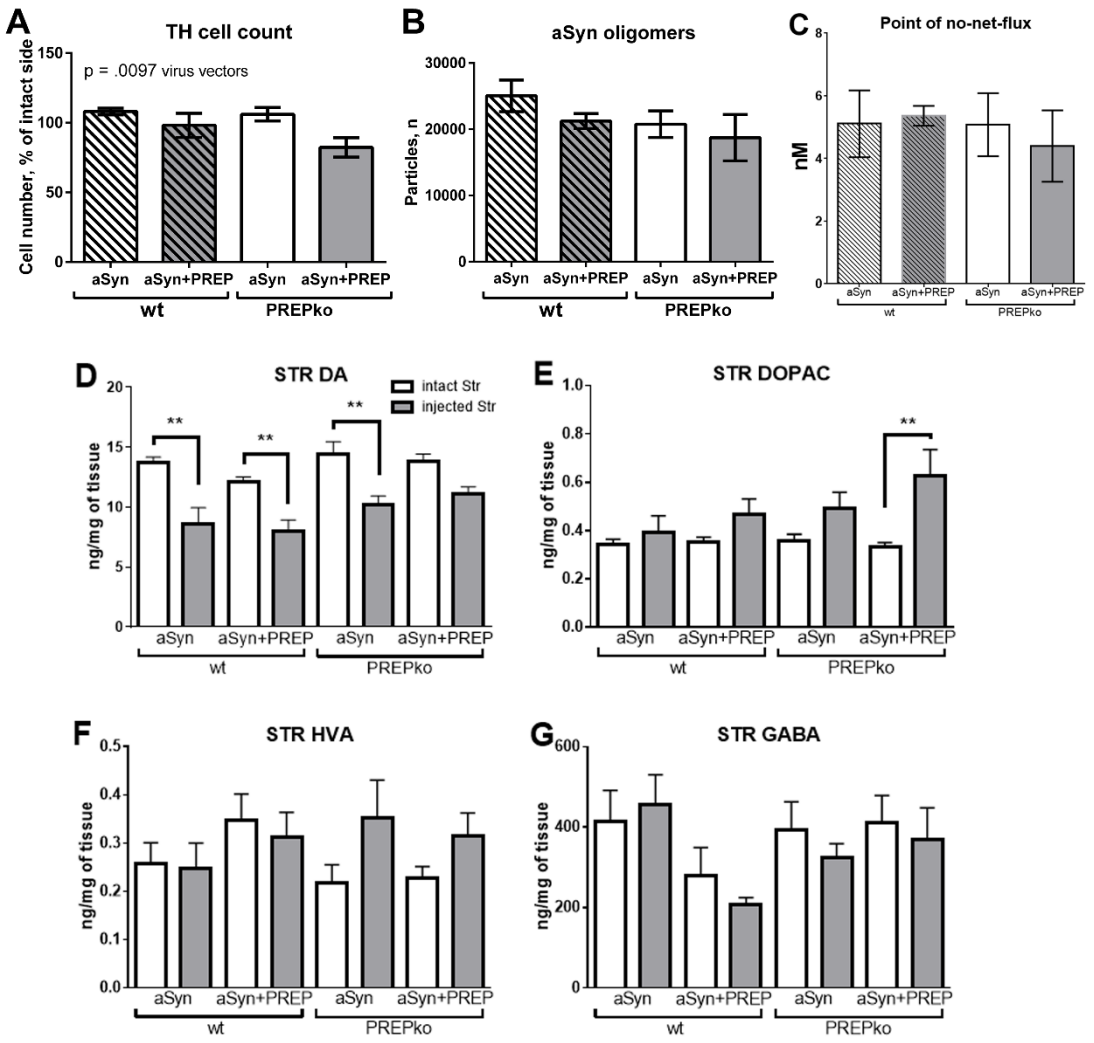

Figure 15 TH positive cells and aSyn oligomers in the SN, striatal extracellular DA, and tissue concentrations of DA, its metabolites and GABA in wild-type (wt) and PREPko mice that received supranigral injection of AAV-aSyn or co-injection of AAV-aSyn and AAV-PREP. TH+ cells were decreased in the SN of aSyn+PREP injected wild-type and PREPko mice compared to aSyn injected wild-type and PREPko mice (A). Number of aSyn oligomer particles (B) and extracellular DA concentration (C) were similar in all groups. Overexpression of aSyn decreased striatal DA concentration in all other groups except aSyn+PREP co-injected PREPko mice (D) but DOPAC was elevated only in this group (E). Tissue concentrations of HVA (F) and GABA (D) were not altered. n = 6-9. Bars represent mean ± SEM. **p < 0.01, two-way ANOVA (A-C) or one-way ANOVA with Tukey´s post-hoc test (D-G).

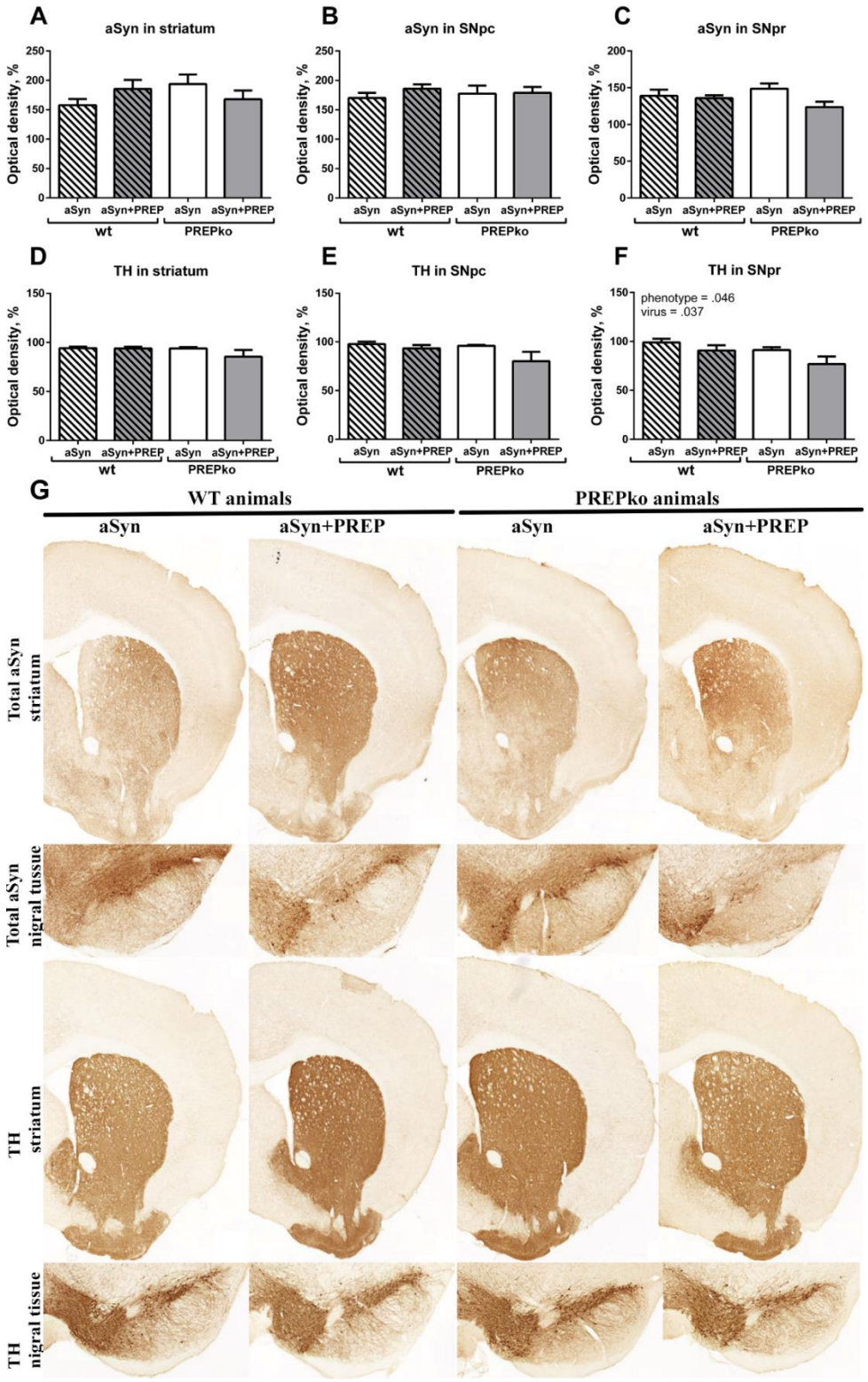

Figure 16 TH and total aSyn in wild-type (wt) and PREPko mice that received supranigral injection of AAV-aSyn or co-injection of AAV-aSyn and AAV-PREP. OD of aSyn was not changed statistically significantly in the STR (A), SNpc (B) or SNpr (C) but there was a trend for decreased aSyn in the SNpr of PREPko mice that received aSyn+PREP co-injection (C). TH+ fibers in the STR (D) and SNpc (E) were not changed but there was a minor decrease of TH+ fiber density in PREPko mice that received aSyn+PREP co-injection that correlated with the TH+ cell count in Figure 15. Representative brain sections from the STR and SN (G). n = 7-8. Bars represent mean ± SEM, two-way ANOVA.

Table 2. *The main findings of the effect of PREP on DAergic neurotransmission.*

DAergic function	**PREP deletion (Studies I + II)**	**PREP overexpression (Studies I + II)**	**PREP inhibition + aSyn overexpression (Study III)**	**PREP deletion + aSyn overexpression (Study IV)**	**PREP + aSyn overexpression (Study IV)**
DA synthesis	Increased by elevated TH in the SN ?	No effect	No effect	Not studied	Not studied
DA storage vesicles	Not studied	Not studied	Restoration?	Protection?	Impaired?
DA release/ SNARE-complex	No effect	Not studied	Restoration?	Protection?	Impaired?
Extracellular DA	Elevated	No effect	No effect	No effect	No effect
Tissue DA	No effect	No effect	No effect	No effect	No effect
DA uptake and DAT phosphorylation	Phosphorylation increased, uptake decreased	Phosphorylation decreased	Not studied	Not studied	Not studied
DAergic cell loss in the STR and the SN	Not studied	Not studied	No effect	Less cell loss than with PREP	More cell loss than without PREP

6 DISCUSSION

6.1 THE ROLE OF PREP IN THE REGULATION OF DOPAMINE TRANSPORTER PHOSPHORYLATION AND FUNCTION (I, II)

PREP has been suggested to participate in the regulation of midbrain DAergic function for the first time when PREP inhibitor S-17092 treatment showed beneficial effects on the cognitive behavior of MPTP-treated monkeys, but the function of the nigrostriatal DAergic system was not studied (Schneider *et al.* 2002). Thereafter, PREP has been shown to participate in the regulation of nigrostriatal DAergic function in the mouse and rat brain (Jalkanen *et al.* 2012, Savolainen *et al.* 2014). PREP inhibitor treatment has been shown to modulate extracellular DA concentration in the rat STR (Jalkanen *et al.* 2012), and tissue concentration of DA and its metabolites in the STR of aSyn transgenic mice (Savolainen *et al.* 2014). PREP inhibitor KYP-2047 was also able to decrease immunoreactive DAT in the STR of transgenic mice (Savolainen *et al.* 2014) indicating that PREP could regulate DAergic neurotransmission by modulating DAT function. Based on these findings we decided to characterize the role of PREP in the nigrostriatal DAergic pathway further. Since PREP inhibitors have shown beneficial effects on aSyn aggregation in *in vitro* and *in vivo* models of PD (Savolainen *et al.* 2014, Myöhänen *et al.* 2012), we wanted to investigate if PREP inhibitor treatment could have a neuroprotective effect on the DAergic neurons in PD models.

In study I, the role of PREP in DAT function was characterized in DAT transfected wild-type and PREPko HEK-293 cells, and, in study II, in PREPko mice and wild-type littermates. The effect of PREP overexpression or restoration of PREP expression was also studied in wild-type and PREPko mice that received supranigral microinjection of AAV-PREP, and in cells that were transfected with a combination of DAT and PREP. AAV-GFP was injected to the control mice, but it turned out that AAV-GFP was not an optimal control viral vector since it showed some toxicity in the behavioral tests in study II. AAV-GFP-induced toxicity has also been reported by other research groups (Albert *et al.* 2018, Landeck *et al.* 2017, Andersen *et al.* 2018), and GFP-induced toxicity could have been avoided by using an empty vector as a control (Andersen *et al.* 2018).

6.1.1 PKC AND ERK IN PREP-MEDIATED REGULATION OF DOPAMINE TRANSPORTER (I)

PKC-mediated internalization is one of the main regulatory mechanisms for DAT function (Vaughan *et al.* 1997, Pristupa *et al.* 1998). PKC is able to phosphorylate and activate ERK, and PREP participates in the regulation of

ERK phosphorylation (Moreno-Baylach *et al.* 2011, Tenorio-Laranga *et al.* 2013) indicating that PREP could possibly regulate DAT function via an ERK- and PKC-mediated mechanism. We studied the role of ERK in DAT transfected wild-type and stable PREPko HEK-293 cells to investigate if lack of PREP, or overexpression, inhibition or restoration of PREP function has an effect on ERK and DAT phosphorylation and if PREP-mediated DAT phosphorylation is dependent on phosphorylation of ERK. The role of PKC was evaluated by measuring DAT and ERK phosphorylation by Western blotting after PREP inhibitor treatment without and in combination with PKC activators and inhibitors, and by measuring PKC activity.

The pDAT antibody used in this study detects the Thr53 phosphorylation site of DAT in the MAPK/SH3 domain (Foster *et al.* 2012) which is phosphorylated by not only ERK but also other MAPKs (Gorentla *et al.* 2009). Thr53 phosphorylation has been shown to regulate kinetics of DAT function by stabilizing DAT on the plasma membrane and increasing uptake of DA (Challasivakanaka *et al.* 2017, Foster *et al.* 2012, Gorentla *et al.* 2009). Surprisingly, deletion of PREP had a trend to increase the phosphorylation of the Thr53 site, although ERK phosphorylation and DA uptake were not significantly changed in study I. This suggests that PREP-regulated phosphorylation of Thr53 is not mediated by activation of ERK. Interestingly, amphetamines have a similar effect on DAT since they also promote internalization of DAT even though they induce phosphorylation of Thr53 (Challasivakanaka *et al.* 2012, Challasivakanaka *et al.* 2017). Additionally, the effect of PREP on DAT function was not observed in the ^{3}H-DA uptake assay even though phosphorylation of Thr53 has been shown to increase plasma membrane localization of DAT and increase DA uptake (Foster *et al.* 2012). Overall, the differences were small in the DA uptake assay suggesting that DAT function was impaired in all groups which was probably caused by the low plasma membrane localization of DAT observed in the immunofluorescence staining. On the other hand, one possibility is that the pDAT antibody also recognizes other phosphorylation sites of DAT than the Thr53 site, which could provide an explanation to the increased phosphorylation of DAT by PKC activator in study I. Consequently, these findings suggest that PREP also regulates DAT function with some other mechanism at the same time with Thr53 phosphorylation. Other possible mechanisms could be e.g. modulation of other phosphorylation sites or PP2A-mediated mechanisms since PREP has been shown to regulate PP2A activity (Svarcbahs 2019) and PP2A has been shown to participate in regulation of DAT (Yang *et al.* 2018). Measuring phosphorylation of the PKC domain and the CaMKII domain located phosphorylation sites of DAT would have provided more information of PREP-mediated DAT phosphorylation and internalization mechanisms, but there were not sufficient antibodies available for those phosphorylation sites. Additionally, regulation of other post-translational modifications, such as palmitoylation, ubiquitination, and glycocylation, could be possible mechanisms in PREP-mediated regulation of DAT function.

Taken together, our studies proved the interaction between PREP and DAT, but since the regulation of DAT by PREP is not dependent on PKC and ERK, unlike predicted, the mechanism of PREP-mediated regulation needs to be studied further.

6.1.2 REGULATION OF DOPAMINERGIC SIGNALING BY PREP *IN VIVO* (II)

In study II, the striatal DAergic function of PREPko mice was studied by no-net-flux microdialysis, fast-scan cyclic voltammetry, and Western blotting analysis of TH and DAT. Elevated extracellular DA level and delayed DA uptake in the STR of PREPko mice pointed to reduced re-uptake of DA by DAT in PREPko mice. Increased Thr53 phosphorylation of DAT suggested changes in DAT function as well. Similar results of DAT phosphorylation and function were observed in the cellular experiments in study I, verifying that phosphorylation of the Thr53 site of DAT correlates with internalization and reduction of DAT-mediated DA transport even though previous studies have shown the opposite result: that Thr53 phosphorylation is stabilizing DAT on the plasma membrane (Foster *et al.* 2012). Additionally, nigrostriatal PREP overexpression induced a decrease in Thr53 phosphorylation which also confirms that PREP regulates the phosphorylation.

Elevated TH expression in the SN of PREPko mice indicates possibly increased DA synthesis, but DA synthesis by TH is also dependent on TH activity, not only TH expression level, and TH activity was not measured in our study. Phosphorylation of TH is the main regulator for its activity (Ramsey *et al.* 1996), and aSyn is able to reduce TH activity by regulating the methylation of PP2A (Perez *et al.* 2002, Peng *et al.* 2005, Hua *et al.* 2015). Since PREP has been shown to interact directly with PP2A and regulate its activity (Svarcbahs 2019), PREP could possibly modify TH activity by modulating PP2A. PP2A also dephosphorylates recently found phosphorylation site Thr48 of DAT which upregulates DAT function (Yang *et al.* 2018) suggesting that PREP could also modulate DAT activity via PP2A.

6.2 THE EFFECT OF PREP DELETION AND INHIBITION ON α-SYNUCLEIN-INDUCED CHANGES IN DOPAMINERGIC SIGNALING (I, III, IV)

aSyn regulates DAergic neurotransmission via several mechanisms by modulating DA synthesis, storage, release, and metabolism (Butler *et al.* 2017). Several studies have shown that aSyn interacts with DAT by direct binding (Lee *et al.* 2001, Wersinger & Sidhu 2003), but also through other mechanisms. However, there have been controversial results as to whether aSyn stabilizes DAT on the plasma membrane (Lee *et al.* 2001, Butler *et al.* 2015) or induces its internalization (Oaks *et al.* 2013, Kisos *et al.* 2014, Swant

et al. 2011, Pelkonen *et al.* 2013). PREP directly interacts with aSyn and regulates its aggregation and oligomerization in cells (Brandt *et al.* 2008, Dokleja *et al.* 2014) and in transgenic mice (Myöhänen *et al.* 2012, Savolainen *et al.* 2014). Since studies I and II showed that PREP participates in the regulation of DAergic neurotransmission, we wanted to study if aSyn is able to modulate the effect of PREP on DAergic function. Additionally, we studied if PREP inhibition, lack of PREP or restoring PREP function modify the effect of aSyn overexpression in cells in study I and in the nigrostriatal DAergic pathway of mouse in studies III and IV. The effect of PREP inhibitor treatment was studied in aSyn overexpression model of PD in study III.

6.2.1 α-SYNUCLEIN AND PREP IN REGULATION OF DOPAMINE TRANSPORTER PHOSPHORYLATION IN CELLS (I)

aSyn regulates DAT function by direct binding to the N-terminal of DAT (Lee *et al.* 2001), but aSyn also inhibits PKC activity (Ostrerova *et al.* 1999) suggesting that aSyn could possibly regulate DAT phosphorylation via PKC. Since PKC activates ERK, the interaction between aSyn and PKC could affect phosphorylation of ERK and DAT. However, we did not observe aSyn-induced alteration in phosphorylation of DAT or ERK in wild-type HEK-293 cells in study I, but aSyn and combination of aSyn and PREP lowered phosphorylation of DAT in the PREPko cells in the Western blotting experiment. However, phosphorylation of ERK was not changed suggesting that aSyn is, similar to PREP, able to modulate phosphorylation of the Thr53 site of DAT independent from ERK phosphorylation even though ERK is known to phosphorylate the Thr53 site at least *in vitro* (Gorentla *et al.* 2009).

aSyn overexpression reduces PKC activity (Ostrerova *et al.* 1999) suggesting that it could reduce phosphorylation of the PKC domain of DAT which could decrease internalization of DAT and increase DA uptake. Nevertheless, phosphorylation of the Thr53 site has the opposite effect (Challasivakanaka *et al.* 2017, Foster *et al.* 2012). A mild decrease in DAT function in a DA uptake assay in aSyn and in aSyn+PREP transfected cells correlates with Thr53 phosphorylation of DAT. This is in line with the previous study showing Thr53 phosphorylation increasing plasma membrane localization of DAT (Foster *et al.* 2012), but opposite to the findings with naïve PREPko cells and PREPko mice in studies I and II. This indicates that although both aSyn and PREP regulate Thr53 phosphorylation of DAT, there are also other simultaneous mechanisms in PREP- and aSyn-mediated regulation of DAT function and localization.

6.2.2 PREP INHIBITOR TREATMENT RESTORES NIGROSTRIATAL DOPAMINERGIC FUNCTION IN MOUSE MODEL OF PARKINSON´S DISEASE (III)

aSyn overexpression induced by supranigral injection of AAV-aSyn significantly reduced extracellular DA, tissue concentration of DA, and TH immunoreactivity in the STR and SN which is in line with the previous studies of an aSyn overexpression model of PD (St Martin *et al.* 2007, Dong *et al.* 2002, Ulusoy *et al.* 2010). PREP inhibitor treatment had a significant improvement on the behavior in the cylinder test although extracellular DA, tissue concentration of DA, and immunoreactive TH in the nigrostriatal pathway were not increased. However, the reduction of toxic aSyn oligomers correlated with the improved behavior. aSyn oligomers have been shown to impair synaptic vesicle function and SNARE-complex formation by binding to the lipid bilayer of the synaptic vesicles and to VAMP2 (Choi *et al.* 2013, Lai *et al.* 2014, DeWitt & Rhoades 2013). These interactions are able to reduce DA release from the presynaptic vesicles to the synaptic cleft. PREP inhibitor-induced reduction of oligomers could possibly enhance DA storage and release by normalizing synaptic vesicle function and SNARE-complex assembly although the extracellular or tissue concentration of DA had not been recovered. aSyn overexpression caused only a minor loss of TH+ neurons thus recovery on neuronal loss is probably not needed to repair the behavioral deficit and DAergic function. A similar phenomenon has also been observed earlier by another research group when aSyn overexpression caused impaired behavior in cylinder test and amphetamine-induced and apomorphine-induced rotation tests but only a mild loss of DA neurons (Gaugler *et al.* 2012). They found aSyn-induced depletion of synaptic vesicles in DAergic axons by quantitative ultrastructural analysis, and reduction in depolarization-induced DA release by amperometric recording, indicating that impaired DA release in the STR has a more remarkable role in behavioral deficits than neuronal cell loss (Gaugler *et al.* 2012). Our similar results indicate that there are similar mechanisms in PREP inhibitor-mediated restoration of DAergic function and behavior.

Autophagy induction reduces DA release by decreasing DA containing synaptic vesicles (Hernandez *et al.* 2012, Limanaqi *et al.* 2018), and PREP inhibition is able to induce autophagy (Savolainen *et al.* 2014) suggesting that PREP inhibition could decrease DA release by autophagy induction. Reduced DA in the presynaptic terminals could also decrease DA-induced oxidative stress and reduce toxicity and cell loss.

6.2.3 DELETION OF PREP PROTECTS FROM α-SYNUCLEIN INDUCED TOXICITY IN MICE (IV)

Although extracellular DA concentration was significantly reduced in the naïve PREPko mice compared to wild-type littermates in study II, a similar difference was not observed in the AAV-aSyn injected STR regardless of

absence, presence, overexpression or restoration of PREP in study IV. PREP was shown to modulate extracellular DA level by controlling DAT function without aSyn overexpression, but an excessive amount of aSyn probably has a greater influence on DAT function than PREP since aSyn directly binds to DAT (Lee *et al.* 2001) and also modulates other regulators of DAT function (Butler *et al.* 2017). Also, the cellular experiments in study I showed that in the presence of aSyn PREP does not have a significant effect on the DAT function.

Deletion of PREP protected mice from behavioral changes in the locomotor activity test and in the cylinder test suggesting that lack of PREP could have a similar protective mechanism as PREP inhibitor treatment. Since extracellular and tissue concentration of DA were not significantly different, the harmful effect of aSyn and combination of aSyn+PREP in wild-type mice could be caused by aSyn-induced reduction in DA storage vesicles and DA release (Choi *et al.* 2013, DeWitt & Rhoades 2013). Deletion of PREP prevented aSyn-induced behavioral deficits suggesting that PREP is needed for aSyn-induced impairment of DA storage and release. Although, restoration of PREP expression to PREPko mice did not cause behavioral changes in the cylinder test indicating some compensative mechanisms in PREPko mice.

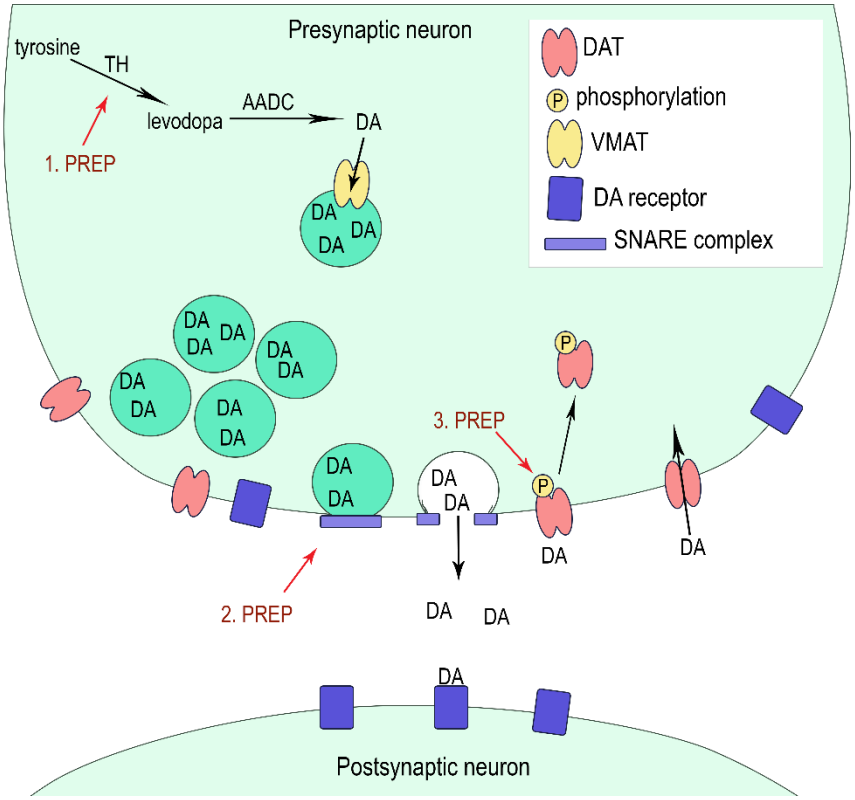

Figure 17 PREP-regulated functions in DAergic signaling. 1. Lack of PREP increased TH in the mouse SN indicating that PREP could decrease DA synthesis by decreasing TH. 2. PREP inhibition and deletion were able to normalize aSyn-induced impairment of DA release and SNARE-complex assembly in mouse brain. 3. PREP regulated DAT function by modulating phosphorylation and internalization of DAT in cells and *in vivo*.

Altogether, aSyn-induced changes in DAT function, DA storage, and DA release seem to cover possible PREP-mediated changes in DAT function. The effect of PREP inhibition on aSyn in study III appears to be different from the effect of deletion of PREP in study IV. Additionally, in study II PREP inhibition failed to produce a similar effect on DAT function as PREP deletion. These findings indicate that there might be compensative mechanisms in PREPko animals and in PREPko cells. Only PREP inhibitor KYP-2047 was used in the studies but other inhibitors could possibly have different effects on DAergic function since protein-protein interactions of PREP are not dependent on PREP enzyme inhibition (Savolainen *et al.* 2015, Svarcbahs 2019). PREP-regulated functions DAergic signaling have been presented in Figure 17.

6.3 FUTURE DIRECTIONS AND SUMMARY OF DISCUSSION

Our studies have shown that PREP certainly has a role in the regulation of the nigrostriatal DAergic system but the mechanisms behind these findings needs to be studied further to be able to utilize the findings in the treatment of PD and other neurodegenerative diseases. Studies III and IV are suggesting that the lack of PREP and PREP inhibition could enhance or restore DA recycling, but this was not measured directly. This could be confirmed for example by measuring density and number of DAergic vesicles in the nerve terminals by quantitative ultrastructural analysis (Gaugler *et al.* 2012) to be able to claim that PREP inhibitor treatment could be beneficial for DAergic function in PD. Study I revealed that PREP-mediated regulation of DAT is independent of ERK and PKC but the actual mechanism has still remained obscure, creating a need for further studies to discover the mechanism. PREP has been shown to regulate PP2A (Svarcbahs 2019) which participates in the regulation of DAT (Yang *et al.* 2018), suggesting that PREP could regulate DAT via a PP2A-mediated mechanism, but this has not yet been studied. There are also numerous other possible targets for PREP-mediated regulation of DAT function if the mechanism appears to be independent from PP2A.

If the PREP-mediated mechanisms in DA recycling and in regulation of DAT can be clarified, PREP inhibitors could provide a novel possibility to treat PD. An advantage in PREP inhibitor-based treatment is that in addition to enhancing dopaminergic function, it can also accelerate autophagy and enhance clearance of aSyn aggregates (Savolainen *et al.* 2014, Svarcbahs 2019), thus making it a multi-target treatment which could modify the progress of PD. This would be an exceptional advancement in the treatment of PD since current medications relieve symptoms, but they are not able to slow or stop the progression of PD. However, it still requires years of preclinical and clinical studies to confirm if PREP inhibitors could have a clinical potential to treat PD or other neurodegenerative diseases.

There has been debate on the role of aSyn in the pathophysiology of PD since some studies have shown that aSyn aggregates and Lewy bodies are non-toxic to the neurons, and Lewy bodies are also found in neurologically normal brains (Parkkinen *et al.* 2005). It has been suggested that aSyn could be an innocent by-stander, and some other mechanism is the main reason for neurodegeneration in PD. However, the role of aSyn in regulation of DAergic homeostasis indicates that misfolded aSyn disturbs normal DAergic function and there are numerous studies showing that oligomeric aSyn has toxic properties (Bengoa-Vergniory *et al.* 2017). PD-linked mutations in SNCA gene are also a strong statement for the importance of aSyn in PD pathophysiology. Therefore, the central role of aSyn does not rule out the possibility for some other fundamental mechanisms in the pathophysiology of PD.

In addition to PREP inhibitors, there are also other aSyn-targeting treatments that are already in clinical trials. A broad-based tyrosine kinase inhibitor, nilotinib, reverses DAergic cell loss and restores motor function by inducing autophagic degradation of aSyn in preclinical models of PD (Hebron *et al.* 2013), and phase I clinical studies have had promising results (Pagan *et al.* 2019). Additionally, aSyn antibody treatments have been able to rescue DAergic cells and improve behavior in animal models of PD (Masliah *et al.* 2005, Games *et al.* 2014), and they also have been safe in phase I clinical trials (Schenk *et al.* 2017). However, phase II clinical trials of aSyn-targeted therapies have not been published so far. If PREP inhibitors or other aSyn-targeted treatments appear to be an effective and safe way to treat PD in the future, they could be the first disease-modifying treatments for PD which could revolutionize the lives of millions of PD patients around the world.

PD is becoming increasingly common since the population is ageing and the duration of the disease has also become longer (Dorsey *et al.* 2018). Additionally, prevalence of PD has been increasing faster than the prevalence of any other neurodegenerative disease in the world. The long duration and the progressive disabling nature of the disease also makes it extremely expensive for society, and it is also important to remember the heavy burden of the disease for an individual patient. This situation creates an emerging need for new and more effective treatments for PD.

7 CONCLUSIONS

The aim of the study was to characterize the role of PREP and aSyn in DAergic neurotransmission in the nigrostriatal pathway. The role of PREP was studied by comparing DAergic function of PREPko cells and PREPko mice. The effect of PREP inhibitor treatment was investigated in an aSyn overexpression model of PD and the role of aSyn was studied in transfected cells and in AAV-injected mice. The main conclusions were:

I PREP regulated DAT phosphorylation and function in transfected HEK-293 cells and in the mouse STR, but the mechanism was independent from PKC and ERK activation.

II PREP inhibitor KYP-2047 treatment restored DAergic function and behavior by decreasing aSyn oligomers, but it did not have a significant effect on the nigrostriatal DA level in an aSyn overexpression model of PD.

III Lack of PREP protected from AAV-aSyn-induced toxicity in the behavioral test although tissue concentration of DA was decreased and the extracellular DA was not altered.

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